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# The development and use of combinatorial glycoarrays to investigate anti-glycolipid antibodies in neurological disease

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A thesis submitted in fulfilment of the requirements of the  
University of Glasgow for the degree of Doctor of Philosophy

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## Dedication

I dedicate this thesis to my parents, Michael and Patricia.

## Acknowledgements

Initially I would like to thank Dr Colin O’Leary for making this whole project possible. Thanks go to Prof Hugh Willison, my supervisor, who was always available and full of encouragement. To his talented bunch at the GBRC- Kate MacDonald, Kay Greenshields, Simon Rinaldi, Rhona McGonigal, Cesc Galban, Sue Halstead- a big thank you for all your help in initiating me into the ways of the laboratoire and throughout my foray into the land of research! To Ariel Arthur- thank you for your beautiful histology pictures and the most excellent chat. To Greg Owens of Denver, Colorado- thank you for the wonderful collaboration. To Gabriela Kalna- thank you for all your help with the heatmaps & cluster analysis.

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## Abstract

The observation that *cis* interactions of neighbouring gangliosides could influence the binding capabilities of anti-glycolipid antibodies has revolutionised the glycolipid world. The realisation of the importance of these interactions has necessitated a novel platform to be developed to assay antibody to a high number of potential glycolipid antigens and their combinations (complexes). I have developed a combinatorial glycoarray technique to assay serum anti-glycolipid and anti-glycolipid complex antibodies and investigated their frequency in two populations of patients with peripheral neuropathy, Guillain Barré Syndrome and Chronic Inflammatory Demyelinating Polyneuropathy. I have compared this technique to the standardized well established technique of assaying glycolipids antibodies, namely enzyme-linked immunosorbent assay (ELISA). In addition I have employed this platform to illustrate the presence of anti-lipid antibodies within the cerebrospinal fluid (CSF) of Multiple Sclerosis (MS) patients. Furthermore through collaboration with other investigators I have demonstrated that oligoclonal immunoglobulin bands (OCB) present in the CSF of MS patients are lipid reactive. This is the first time since their original description over 50 years ago that the specificity of MS derived OCB has been described. Whilst the pathological significance of these lipid reactive antibodies remains uncertain their description provides new avenues for future research.

## Declaration

The results presented in this thesis are my own work except where there is an explicit statement to the contrary.

Kathryn M. Brennan

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## Abbreviations:

AGA	anti-ganglioside antibody
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AMAN	acute motor axonal neuropathy
AMSAN	acute motor and sensory axonal neuropathy
BoNT	<i>Clostridium botulinum</i> neurotoxin
BSA	bovine serum albumin
Cardio	cardiolipin
Cer	ceramides
Chol	cholesterol
CIDP	chronic inflammatory demyelinating polyradiculoneuropathy
CIS	clinically isolated syndrome
CMAP	compound muscle action potential
CNS	central nervous system
CSF	cerebrospinal fluid
CVs	coefficients of variance
DGG	digalactosyl diglyceride
EAN	experimental autoimmune neuritis
EAE	experimental autoimmune encephalitis
ECL	enhanced chemiluminescence substrate
ELISA	Enzyme linked immunosorbent assay
Fab	fragment antigen binding region of antibody
FAFBSA	fatty acid free bovine serum albumin
GalC	galactocerebroside
GBS	Guillain-Barré syndrome
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GSC	anti-ganglioside complex (antibodies)
HC	healthy controls
HIS	polyhistidine

HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IgG	immunoglobulin
IVIG	intravenous immunoglobulin
LOS	lipo-oligosaccharide
LPS	lipopolysaccharide
MAG	myelin associated glycoprotein
MCV	motor conduction velocity
MFS	Miller Fisher syndrome
MGG	galactosyl diglyceride
MMN	multifocal motor neuropathy
MS	multiple sclerosis
MV	measles virus
MRC	Medical Research Council
MRI	magnetic resonance imaging
NeuAc	sialic acid, N-acetylneuraminic acid
NMO	neuromyelitis optica
OCB	oligoclonal band
OIND	other inflammatory neurological disease
OND	other neurological disease
ONLS	overall neuropathy limitation scale
PBS	phosphate buffered saline, Ph 7.2
PC	L alphaphosphatidylcholine
PE*	L alphaphosphatidylethanolamine
PE	plasmapheresis
PIP(4)	L alphaphosphatidylinositol-4-phosphate
PL	plasmalogen
PNS	peripheral nervous system
PPMS	primary progressive multiple sclerosis
PS	L alphaphosphatidylserine
PVDF	polyvinylidene fluoride
rAb	recombinant antibody
RIA	Radioimmunoassay

RRMS	relapsing remitting multiple sclerosis
SDS	sodium dodecyl sulphate polyacrylamide gel
SEM	standard error of the mean
Siglecs	sialic acid binding immunoglobulin like lectins
SM	sphingomyelin
SPMS	secondary progressive multiple sclerosis
SS	sphingosine
SSPE	subacute sclerosing panencephalitis
Sulf	sulfatide
TLC	Thin layer chromatography
VSV	varicella virus
w:w	weight:weight

## Chapter 1. Introduction

### 1.1. Lipids

Lipids are fatty acids and their derivatives. They have long been recognised as key components in the bilayer of cell membranes and are involved in many diverse roles including energy storage, cell signalling and trafficking (Posse de Chaves et al. 2010). Autoimmunity against lipids is noted in a wide variety of central and peripheral nervous system diseases (Plomp et al. 2009) (Kanter et al. 2006)(Yamazaki et al. 2008)(Lepur et al. 2007). The role of such autoimmunity to lipids is still unclear in a number of these disease processes.

The elucidation of the role auto antibodies directed against lipids has been a central theme in neurological disease over the last 50 years particularly in the acute polyradiculoneuropathy Guillain-Barré syndrome. This is an acute self-limiting post infectious neuropathy first described by French physicians during World War 1 (Guillain et al. 1916). A history of preceding infection is common e.g. gastrointestinal or upper respiratory tract with some of the causative microorganisms of these preceding infections been shown to have carbohydrate structures similar to those of glycolipids (or gangliosides) which are prominent in the peripheral nervous system suggesting molecular mimicry as a pathological basis for the disease process (Ang et al. 2004, Yuki et al. 2006). These anti-ganglioside antibodies may be pathogenic and result in the development of neurological disability (Plomp et al. 2009, Willison et al. 2008).



## 1.2. Glycolipids and gangliosides

### 1.2.1 Ganglioside structure and synthesis

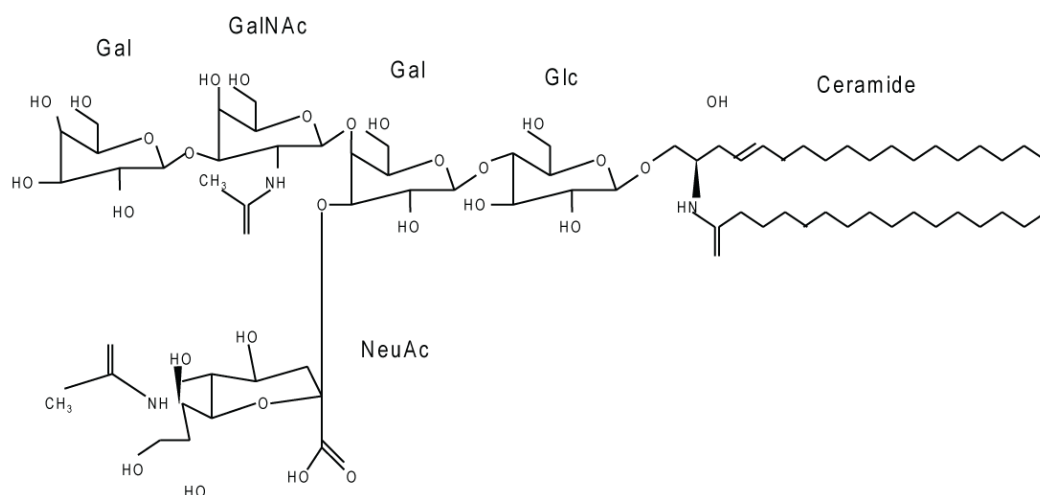
Glycolipids simply are lipids which have a carbohydrate chain attached. Gangliosides are sialic acid bearing glycosphingolipids. Sialic acid refers to any member of the diverse family of nine-carbon neuraminic acid-based sugars. In humans, sialic acids are predominantly *N*-acetylneuraminic acid, abbreviated “NeuAc”. Gangliosides are ubiquitously expressed in all vertebrate tissue and are particularly expressed in cell surface membranes in the central and peripheral nervous systems where they contribute up to 10-12% of the total lipid content (Tettamanti et al. 1973). These are amphipathic molecules consisting of a carbohydrate core attached to a ceramide moiety (Figure 1.1 (A)). These membrane lipids are arranged such a manner that the carbohydrate moiety sits on the exoplasmic surface of the cell membrane with the ceramide tail embedded into the bilayer of the cell membrane (Hakomori Si 2002)(Sonnino et al. 2007). The carbohydrate moiety consists of a varying chain of neutral sugars linking one or more sialic acid residues. Subclasses of gangliosides are defined based on the core of four neutral sugars attached to the ceramide.

The nomenclature used is described by Svennerholm, designating gangliosides as G $X$ yz, where G indicates ganglioside,  $X$  represents the number of sialic acid residues, (M=1, D=2, T=3, Q=4),  $y$  indicates the length of the carbohydrate sequence (defined as 5 minus the number of residues) and  $z$  is a letter indicating the isomeric form i.e. reflecting the position and linkage of the sialic acid residues (a, b, c) (Svennerholm 1994). The ganglioside biosynthetic pathway, which takes place in the Golgi complex, is outlined in Figure 1.1 (B). Gangliosides are synthesized by the sequential addition of sialic acids and oligosaccharides to a glycosylceramide molecule

which is catalysed by a series of specific glycosyltransferases (Figure 1.2.)(Maccioni 2007). In addition, located in the plasma membrane is the enzyme “plasma membrane ganglioside sialidase” or neuraminidase 3. This enzyme specifically hydrolyses sialic acid residues from polysialosylated gangliosides to produce GM1 within the plasma membrane (Miyagi et al. 1999) (Wada et al. 1999).

Variation can be further introduced the ganglioside molecule via alterations in the lipid backbone via changes in the length, degree of hydroxylation or saturation of either the sphingoid base or the fatty acid or both (Norton 1977).

A



B

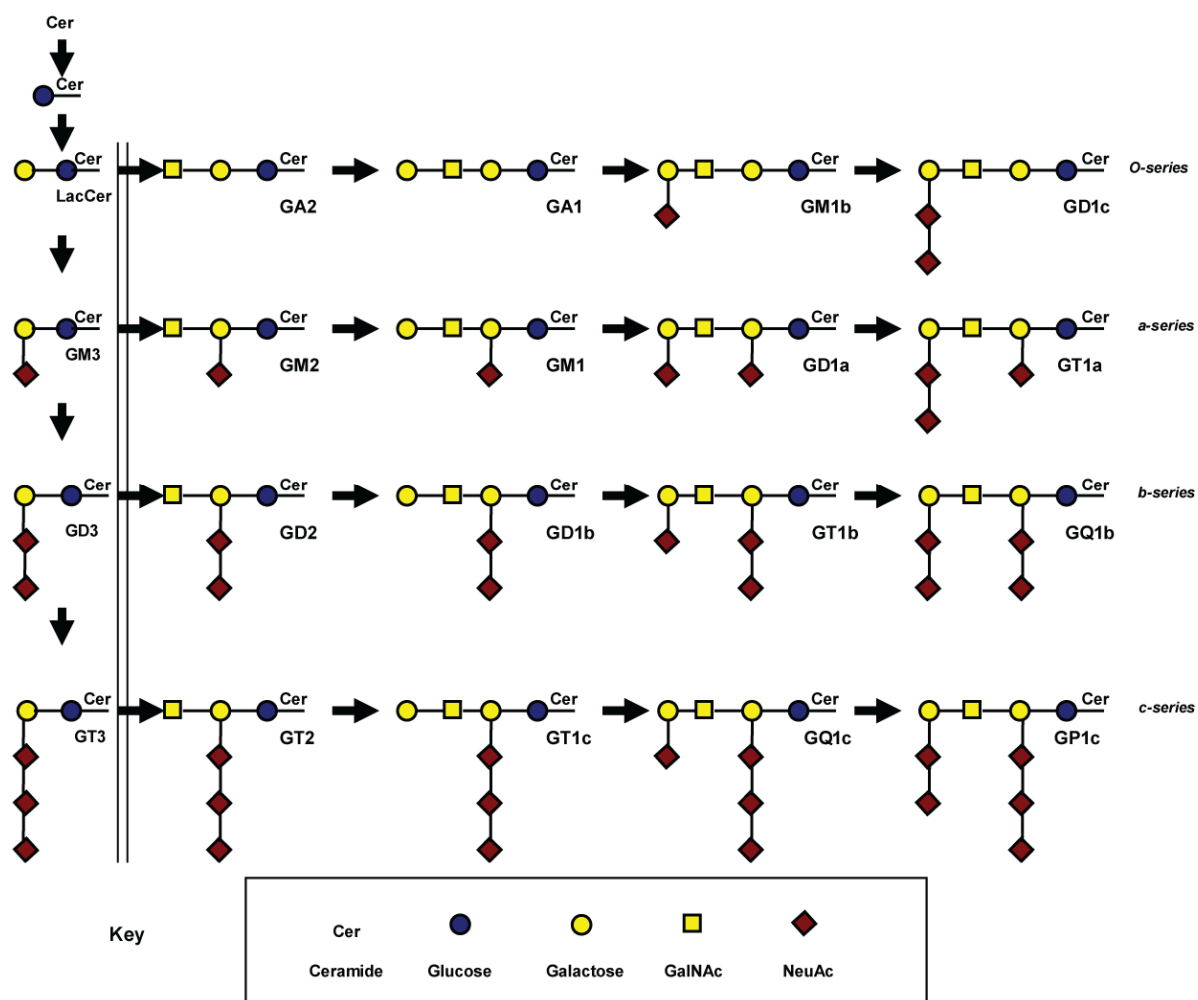


Figure 1.1. The structure and biosynthetic pathway of gangliosides

Key:

GalNAc= N-Acetylgalactosamine

NeuAc= N-Acetylneuraminic acid

A. Structure of the ganglioside GM1

B. The biosynthetic pathway of gangliosides

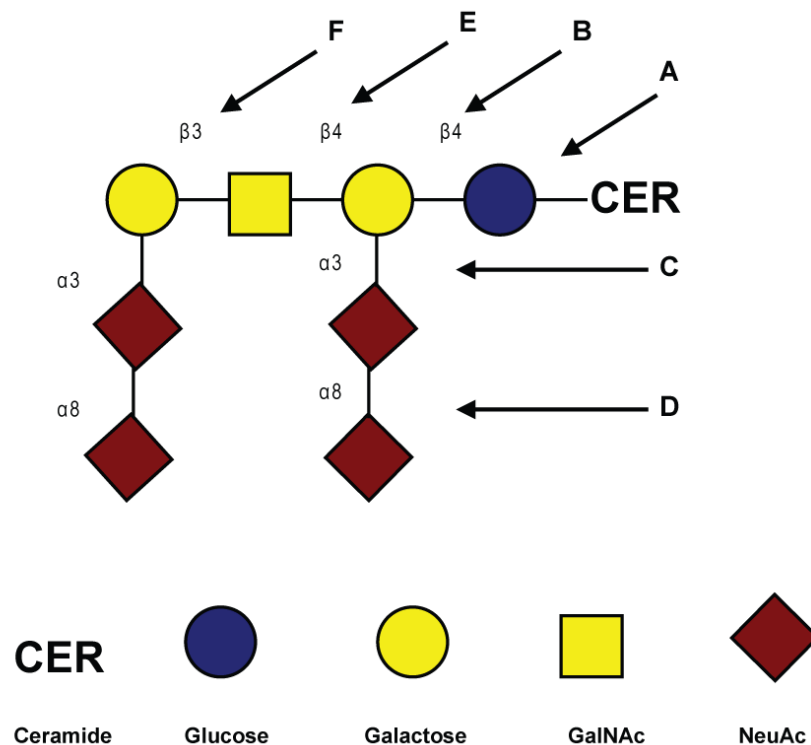


Figure 1.2. The enzymes involved in the biosynthetic pathway of gangliosides

A= ceramide glucosyltransferase

B=  $\beta$ 1,4 galactosyltransferase

C=  $\alpha$  2,3 sialtransferase

D=  $\alpha$  2,8 sialtransferase

E= N-acetylgalactosaminyltransferase

F=  $\beta$  1, 3 galactosyltransferase

### 1.2.2. Ganglioside localisation

Gangliosides are anchored in the outer leaflet of the plasma membrane, where the long hydrocarbon chain of ceramide is embedded into the lipid bilayer allowing the oligosaccharide moiety to project outwards from the cell surface and potentially interact with a number of antigens. Gangliosides are prominent in the cell membranes of neurons and are organised into clusters of fellow lipid such as cholesterol and proteins known as lipid rafts (Simons et al. 2000). Lipid rafts or membrane “detergent resistant” microdomains are dynamic clusters of specific proteins e.g. glycoposphatidylinositol anchored proteins, lipids and kinases that can alter their composition and structure in response to intra- or extracellular stimuli. Recent work suggests that gangliosides may play an active role in the formation of these membrane microdomains (Silveira e Souza et al. 2008) (Sonnino et al. 2007).

### 1.2.3. Ganglioside function

The exact biological functions of gangliosides have not been fully elucidated as yet. The functions modulated by gangliosides postulated include modulation of membrane proteins, neural development, cell-cell interaction/recognition, neuronal  $\text{Ca}^{2+}$  homeostasis, axonal growth, node of Ranvier stability and synaptic transmission (Lopez et al. 2009) (Plomp et al. 2009).

Evidence does exist for the binding of an immunoglobulin like family of proteins known as lectins. These are present on opposing cell membranes and one subtype in particular, the siglecs, bind to sialic acid containing glycans such as gangliosides (Crocker et al. 2007). Within the siglec family,

one member, myelin associated glycoprotein (MAG) is expressed in brain and appears to bind with high specificity to the gangliosides GD1a and GT1b. MAG is membrane glycoprotein that is selectively located in periaxonal Schwann cell and oligodendroglial membranes of myelin sheaths. It is important for the normal formation and maintenance of myelinated axons (Quarles 2007). Both GD1a and GT1b gangliosides share the same terminal sequence or epitope, “NeuAc  $\alpha$ 2-3 Gal  $\beta$ 1-3 GalNAc” (Figure 1.1 (B)). In vitro, MAG binds with high affinity to GD1a and GT1b but not to closely related gangliosides such as GM1 or GD1b which lack the “NeuAc  $\alpha$ 2-3 Gal  $\beta$ 1-3 GalNAc” terminus (Yang et al. 1996) (Collins et al. 1997). This led to the hypothesis that MAG, on myelin, functions in vivo by binding to gangliosides GD1a and/or GT1b expressed on the axon surface (Yang et al. 1996).

Gangliosides appear to participate in axonal development primarily through the modulation of membrane receptor signalling activity (Abad-Rodriguez et al. 2007). This interaction is supported by neurotrophins via signalling via neurotrophin receptors (Trks, p75 neurotrophin receptor (p75<sup>NTR</sup>)). Specific ganglioside-receptor interaction is capable of supporting (GM1-TrkA) or inhibiting axonal growth (GD1a/GT1b-p75<sup>NTR</sup>). These interactions are subjected to fine regulation by the conversion of complex gangliosides to simpler ones (e.g. GD1a to GM1). This particular regulatory process can be spatially confined by local accumulation of ganglioside-converting enzymes such as neuraminidase 3 in the plasma membrane.

Gangliosides have also been demonstrated to act as receptors for bacterial toxins. Both neurotoxins from *Clostridium botulinum* (BoNT) and *Clostridium tetani* (TeNT) use gangliosides as cell surface receptors. TeNT binds with highest affinity to the complex gangliosides GT1b and GQ1b with lesser affinities to GD1a and the monosialated gangliosides (Walton et al.

1988). There are seven different subtypes of BoNT (A-G) with heterogeneity of ganglioside binding profiles observed. However all will bind with highest affinity to GT1b and GQ1b(Kitamura et al. 1980)(Kamata et al. 1986). Although both toxins bind at the motor nerve terminals of the neuromuscular junction, they are associated with different clinical sequelae. TeNT binding also occurs within the central nervous system following retrograde transport from the axon terminal to inhibitory neurons within the spinal cord. This results in the prevention of neurotransmitter release causing the pathognomonic sustained contraction of muscles (Schiavo et al. 1992). In contrast BoNT is active within the peripheral nervous system disrupting neurotransmitter release causing a flaccid paralysis which may be similar clinical picture to acute GBS (Burgen et al. 1949).

Gangliosides also have the ability to function as receptors for viruses such as influenza virus. Infection of cells by this virus is initiated by the binding of haemagglutinin, a viral membrane protein, to sialic acid containing oligosaccharides, such as GM3 (Paulson et al. 1979) (Suzuki et al. 1986). This binding mediates viral entry and membrane fusion.

Neuronal and non-neuronal tissues have different ganglioside profiles which may suggest regional specific functions. The more complex gangliosides are a feature of neuronal cell membranes whereas the more simplistic gangliosides such as GM3 and GD3 are more predominant in non-neuronal tissue such as liver and muscle (Fishman 1974)(Fishman et al. 1976). This difference in complexity of ganglioside profile expressed in neuronal and non-neuronal tissue is presumed to be secondary to the high level of UDP-GalNAc-GM3-acetylgalactosaminyltransferase (GalNAc- transferase) expressed in the cells of the nervous system (Dicesare et al. 1971).

#### 1.2.4. Neurological disease and glycolipids

As aforementioned several neurological diseases are associated with autoimmunity to glycolipids. These include the peripheral neuropathies, Guillain-Barré syndrome and Chronic Inflammatory Demyelinating Polyradiculoneuropathy and the central nervous system demyelinating disorder, Multiple Sclerosis.

### 1.3. Guillain-Barré syndrome

There is considerable data pointing toward an association between anti-glycolipid antibodies and the acute polyradiculoneuropathy Guillain-Barré syndrome (GBS). As gangliosides are highly enriched in cell surface membranes in both the central and peripheral nervous systems they have long been considered to be the likely antigenic target in this neuropathy. Classically this monophasic neuropathy presents with an ascending motor weakness with loss of tendon reflexes with peripheral sensory symptoms. This acquired neuropathy is preceded by an infection in around two-thirds of patients with the commonest observed microbial agent being *Campylobacter jejuni* enteritis (Rees et al. 1995) (Jacobs et al. 1998).

#### 1.3.1. Epidemiology of GBS

GBS has an annual incidence of one or two per hundred thousand people (Alter 1990). The lifetime risk of any one individual acquiring the disease has been calculated to be approximately 1 in 1000.



### 1.3.2. Clinical Features and pathology of GBS

The classical clinical presentation consists of an acute progressive symmetrical motor weakness reaching a nadir within 4 weeks (Van der Meché FG. Van Doorn PA. Meulstee J. Jennekens FG. GBS-consensus group of the Dutch Neuromuscular Research Support Centre 2001). This may be associated with sensory signs and symptoms but these are typically mild. In addition there is a loss of tendon reflexes. Autonomic symptoms affecting bladder, bowel and cardiac function may be associated. However a variety of clinical subtypes are observed with four main subtypes described based on clinical phenotype, pathology and aetiology and include acute inflammatory demyelinating polyradiculoneuropathy (AIDP), acute motor axonal neuropathy (AMAN), acute motor and axonal neuropathy (AMSAN) and Miller Fisher Syndrome (MFS) (Asbury et al. 1997) (Plomp et al. 2009).

AIDP is the most common form in Europe and North America and is characterized by cellular infiltration of T cells and macrophages and subsequent demyelination and axonal damage (Hughes et al. 2005). The axonal forms, AMAN and AMSAN, are only rarely encountered in this demographic. However in Japanese and Chinese populations these axonal variants, which appear to mediate pathology via antibody mediated damage, can account for 10-30% of cases (Yuki 2005). In these axonal forms the primary target appears to be motor and/or sensory nerve axolemmal membrane (Feasby et al. 1986). In AMAN patients axolemmal membranes expressed at nodes of Ranvier and motor axon terminals are targeted, resulting in paralysis secondary to denervation (Ho et al. 1997). The pathology observed in AMSAN closely resembles that seen AMAN with both motor and sensory nerves affected in AMSAN (Griffin et al. 1996). In contrast to AIDP demyelination in the axonal variants is absent or minimal.

Interestingly in addition to the heterogeneity seen in the ganglioside profile of neuronal and non neuronal tissue there is diversity to the ganglioside profile within neuronal tissue. This may explain the observation that the different clinical phenotypes observed in GBS can be associated with specific anti-ganglioside antibody profiles (Kaida et al. 2010). An example is the Miller-Fisher variant of GBS. This triad of ataxia, areflexia and ophthalmoplegia is associated with IgG directed against the complex ganglioside GQ1b (Chiba et al. 1992) (Kusunoki et al. 1999). Biochemical analysis of gangliosides isolated from cranial nerves and spinal nerve roots from humans show that GQ1b is particularly enriched in the oculomotor, trochlear and abducens nerves. Further immunohistochemical studies using a monoclonal antibody directed against GQ1b reveal that the paranodal myelin of the extramedullary portion of these cranial nerves were specifically immunostained (Chiba et al. 1993). Similar immunostaining was observed in a subset of neurons in dorsal root ganglia which may explain the development of ataxia in such patients (Kusunoki et al. 1999).

### 1.3.3. Diagnosis of GBS

GBS is a diagnosis made on both clinical and electrophysiological grounds (Van der Meché FG. Van Doorn PA. Meulstee J. Jennekens FG. GBS-consensus group of the Dutch Neuromuscular Research Support Centre 2001). The major clinical criteria include symmetrical weakness with a decrease or disappearance of distal limb reflexes and reaching a nadir within 4 weeks. CSF protein levels rise with a near normal cell count. Presumably this protein rise is a reflection of the breakdown of the blood brain barrier. Differential diagnoses must be excluded. The electrophysiological findings should support a polyneuropathy and should include 3 of the following abnormal parameters in at least 2 individual nerves (Van der Meché FG. Van Doorn PA. Meulstee J. Jennekens FG. GBS-consensus group of the Dutch Neuromuscular Research Support Centre 2001):

1. Prolonged distal motor latency
2. Reduced nerve conduction velocity
3. Prolonged F-wave latency (given a normal distal conduction velocity) indicating proximal conduction slowing or block
4. Reduced amplitude of the distal compound muscle action potential (CMAP)
5. Abnormal reduction of the CMAP with proximal versus distal stimulation with a distal CMAP above 5mV measured peak to peak
6. Increase in the duration of the CMAP with proximal stimulation versus distal stimulation (temporal dispersion)
7. Reduction in amplitude of the sensory nerve action potential
8. Abnormal recruitment pattern on EMG: either absent or “single pattern”
9. Presence of spontaneous muscle fibre activity

The characteristic features of AIDP include delayed or absent F waves, reduced motor conduction velocities with temporal dispersion and prolonged distal motor latencies. Small well formed CMAPs in the absence of demyelinating features are characteristic of the axonal variants. Sensory nerve action potentials may be reduced or absent in AIDP, AMSAN and MFS.

It is noteworthy that electrophysiological patterns can evolve during the course of the illness. Furthermore when nerves are completely unexcitable classification into subtypes is not possible.

#### 1.3.4. Treatment of GBS

Treatment with either IVIG or plasma exchange have been shown hasten recovery (Hughes RA. Raphaël JC. Swan AV. van Doorn PA 2006) (Raphaël JC. Chevret S. Hughes RA. Annane D 2002). Either treatment when given within 2 weeks of diagnosis, approximately halves the number of patients requiring ventilation and doubles the speed of recovery. There is no evidence for combination therapy and currently no evidence for the use of corticosteroids in GBS (Hughes et al. 2010).

#### 1.3.5. Anti-glycolipid antibodies and GBS

Specific anti ganglioside antibody profiles are associated with distinct clinical phenotypes in GBS. Anti-GM1 IgG is strongly linked to motor axonal forms of GBS with 50-80% of AMAN patients having higher anti-GM1 IgG levels in their serum than normal controls (Jacobs et al. 1996). These antibodies are also observed in the closely related clinical syndrome AMSAN (Hadden et al. 1998). Further studies reveal that AMAN and AMSAN are associated with IgG antibodies against (combinations of) GM1, GD1a or GalNAc GD1a. However what is not clear is the mechanism by which anti GM1 IgG can produce such clinically distinct phenotypes. GM1 is expressed in equal abundance on both sensory and motor nerves and it is, as yet, unclear how a specific anti-ganglioside antibody can be associated with such clinically distinct phenotypes. Anti- ganglioside antibody titres tend to be highest in the acute phase and decrease with clinical improvement suggesting that these anti-glycolipid antibodies may be directly associated with the pathogenic mechanisms.

Whilst there is a well defined relationship between the axonal variants of GBS and anti-gangliosides antibodies, in contrast AIDP has not been consistently found to be associated with a specific anti-ganglioside antibody profile (Plomp et al. 2009). The subtype Miller Fisher syndrome is associated with antibodies directed to the complex ganglioside GQ1b which

is particularly enriched in the nerves supplying the extra ocular eye muscles. This pattern of distribution of the ganglioside GQ1b would appear to correlate with the clinical sequelae associated with Miller Fisher Syndrome and would suggest pathogenicity. Indeed it has been demonstrated in the animal model that anti-GQ1b antibodies bind to motor nerve terminals in ex-vivo preparations of mouse hemi diaphragm subsequently activating complement (Halstead et al. 2004) (Plomp et al. 1999). This then activates the membrane attack complex (MAC) pore formation which leads to unregulated calcium influx, acetylcholine release and neuromuscular transmission block. What is unexplained is the mechanism by which some nerves with high GQ1b concentrations are protected or “immune” to pathology of MFS as observed by Chiba et al (Chiba et al. 1997). Despite the inconsistencies evidence does point toward a strong association between anti-ganglioside antibodies and GBS pathology.

#### 1.3.6. Molecular mimicry

The hypothesis was put forward recently that anti-ganglioside antibodies arise in GBS via molecular mimicry (Ang et al. 2004)(Yuki et al. 2006). Around two thirds of GBS patients develop the syndrome around ten to fourteen days after infection with various agents (Hughes et al. 1999). The most common preceding infection is *Campylobacter jejuni* enteritis, although other bacterial and viral infections, mainly of the respiratory and gastrointestinal tract, have also been implicated (Jacobs et al. 1996) (Lehmann et al. 2010). Both structural and serological studies on strains of *Campylobacter jejuni* isolated from GBS patients, reveal that the oligosaccharide moiety of their lipopolysaccharide (LPS) can mimic the oligosaccharide moieties on gangliosides (Yuki et al. 1993). LPS mimics of the gangliosides GM1, GD1a and GD3 have all been described (Aspinall et al. 1994)(Salloway et al. 1996). In addition antibodies that cross react with gangliosides have been produced when animals have been immunised with

LPS structures with the corresponding oligosaccharide moieties (Goodyear et al. 1999) (Ang et al. 2001) (Bowes et al. 2002).. This is the basis for the theory of molecular mimicry with immunisation of LPS or ganglioside producing antibodies that recognise and bind self antigens.

Although it has not been demonstrated, viruses may acquire gangliosides on their envelopes through their emergence out of infected host cells. This “budding” is a process by which viruses exit infected host cells by enveloping the virus core with host cell membranes. Gangliosides contained in these host cell membranes could then be exposed to immune cells on the virus envelope.

It is unclear what host mechanisms are involved in the determination of an aberrant immune response (manifest by an acute neuropathy) when the host is challenged with an infection such as *Campylobacter jejuni* enteritis. Only 1 in 3000 infected people will develop GBS (Plomp et al. 2009). One review speculates that either the microorganism strain is of a highly specific specialized nature (for example bearing ganglioside like oligosaccharide cores on the lipopolysaccharide backbone) or that the host may have susceptibility factors which increase the likelihood of post-infectious GBS. Or that the propensity to develop GBS may only occur if these two conditions are met. The authors mention that gene variants have been described in carbohydrate-synthesizing enzyme genes of *Campylobacter jejuni* strains which have been isolated from GBS patients. These would then enable the synthesis of ganglioside like oligosaccharide structures to allow molecular mimicry to take place.

### 1.3.7. Anti-glycolipid complex antibodies

The observation that *cis* interactions of neighbouring gangliosides could influence the binding capabilities of anti-glycolipid antibodies was reported recently by Kaida et al (Kaida et al. 2004). When performing TLC immunostaining on gangliosides with serum from a 31 year old with acute flaccid paralysis the investigators noticed binding to a mixture of GD1a and GD1b gangliosides. Further studies using ELISA confirmed higher affinity binding to the complex of lipids compared to each individual lipid introducing the concept of anti-ganglioside complex (GSC) antibodies to the glycolipid world. Further screening of a population of 100 GBS cases revealed the presence of IgG directed against the complex of GD1a and GD1b in 8 cases. The authors report that the anti-GSC reactivity was maximal when 6:4 or 5:5 (w:w) ratios of the two gangliosides were applied. In some cases (3/8) there was no IgG detectable against the contributing lipid in isolation (Figure 1.3.), in others there was detectable but lesser degrees of binding to the contributory lipids. This group of patients with anti-GD1a/GD1b antibody appeared to be predisposed to lower cranial nerve palsy and severe disability however this observation was not statistically significant. At the time the authors defined anti-GCS positivity when the optical density (OD) for the ganglioside complex was 0.2 higher than for either single ganglioside. This definition was revised some time later with anti-GSC's only being defined to be present if the OD for the IgG directed against the complex of lipids was more than the sum of the OD's of IgG directed against each contributing lipid (Kaida et al. 2007).

This further study confirmed the observation that presence of these anti-GSC antibodies could aid with clinical stratification. 234 GBS cases were investigated for the presence of anti-GSC antibodies. 39 (17%) had detectable anti-GSC antibodies using the newer definition. This represented IgG directed against a number of ganglioside complexes including

GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b and GM1/GD1b. This particular subset of patients was more likely to have had an antecedent gastrointestinal infection and lower cranial nerve deficits. Those patients with IgG to the particular complexes of GD1a/GD1b and/or GD1b/GT1b were more likely to have severe disability and require mechanical ventilation (two tailed  $p = 0.009$  and  $0.003$  respectively). The authors went on to search for IgG directed against three or four ganglioside complexes. They observed that the previously observed anti-GSC reactivity was often attenuated in such circumstances leading to the conclusion that heteromeric pairs of glycolipids were sufficient for the creation of IgG binding epitopes.



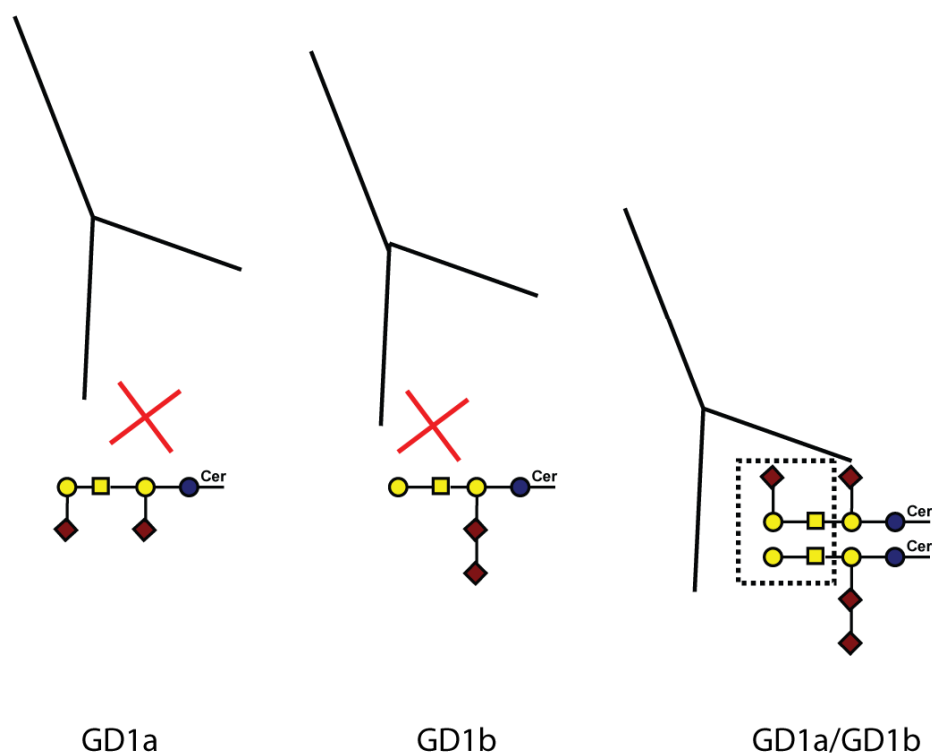


Figure 1.3. Illustration of an anti ganglioside complex (GSC) antibody  
In the first large series of GBS patients screened for anti ganglioside complex antibodies a subset of patients with antibody directed only to the complex of the two lipids GD1a/GD1b was observed(Kaida et al. 2004)

This observation that cis interactions of neighbouring glycolipids could alter the binding profile of IgG is intriguing and not wholly unexpected(Varki 1994). Over 15 years ago the concept that combinations of oligosaccharide groups from different glycans may form a distinct “lectin-type” (selectin) epitope was suggested. This was based on the observation that selectins had unusually high affinity for their target glycans which was unexpected for their monovalent oligosaccharide ligands. The authors hypothesized that the oligosaccharide ligands grouped together to form “clustered saccharide patches” which resulted in high affinity binding. This theory ties in nicely

with the more recently described realisation that lipids are not distributed randomly throughout the plasma membrane but are rather compartmentalized in functional membrane microdomains with the ability to interact with fellow raft lipids, proteins and enzymes (Simons et al. 1997)(Simons et al. 2000). The importance of this local “microenvironment” has ramifications not only in GBS but in other nervous system diseases such as CIDP and Multiple Sclerosis, in which auto antibodies to lipids have been described (Hughes et al. 2006)(Kanter et al. 2006).

#### 1.4. Chronic Inflammatory demyelinating polyradiculoneuropathy

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is an acquired demyelinating disease of the peripheral nervous system (PNS) predominantly affecting spinal roots, major plexuses and proximal nerve trunks(Said 2006).

##### 1.4.1. Epidemiology of CIDP

The prevalence of CIDP in across Japanese and Caucasian populations is reported to be similar (1.24 - 1.9 per 100,000)(Lunn et al. 1999)(McLeod et al. 1999)(Iijima et al. 2008) . Across populations, an increasing prevalence and incidence is noted with age with a male predominance over females reported. These epidemiological similarities suggest that the pathogenesis of CIDP may be common worldwide, and independent of genetic and geographical influences.

##### 1.4.2. Clinical features of CIDP

CIDP is a generalised demyelinating disease characterized by progressive or relapsing weakness and impaired sensory function in the upper and lower limbs(McCombe et al. 1987). It typically is an insidious chronic neuropathy which displays a progressive or relapsing clinical course. Segmental

demyelination is evident in peripheral nerves. This neuropathy encompasses a heterogeneous group including “typical” and “atypical” CIDP (European Federation of Neurological et al. 2006). “Typical” CIDP develops over 8 weeks and is a chronic progressive, stepwise or recurrent symmetrical proximal and distal weakness with sensory dysfunction of all 4 limbs which are hypo/areflexic. “Atypical” CIDP patients may have intact reflexes and can have different patterns of weakness e.g. predominantly distal weakness, pure motor or pure sensory presentations, asymmetrical presentations or focal presentations. Whether these variants respond to specific immunotherapies is largely unknown.

#### 1.4.3. Pathology of CIDP

CIDP is considered to be an autoimmune condition as a significant proportion of patients respond to immunotherapies (van Doorn 2005). Demyelination is a key pathological feature with myelin considered to be the likely target for the immune response. Certain pathological features are diagnostic hallmarks and include “onion bulb” formation, perivascular inflammatory infiltrate with predominant demyelination (Toyka et al. 2003). The “onion bulb” formations of layers of Schwann cell cytoplasm interspersed with collagen fibres and loss of nerve fibres occurs as a result of proliferation of Schwann cell processes. Axonal degeneration is typically a chronic feature. The exact pathogenic mechanisms are unclear however evidence exists for both cellular and humoral mechanisms.

Nerve biopsies have provided evidence for the presence of inflammatory infiltrate in CIDP. This infiltrate, consisting of macrophages and T cells, would suggest that T cell mediated mechanisms are involved (Schmidt et al. 1996). In addition increased levels of inflammatory cytokines and chemokines have been reported in nerves, CSF and serum of CIDP patients together with increased circulating T cells (Lunn et al. 2009).

Antibodies directed to myelin derived lipid or protein antigens have been demonstrated in CIDP. Deposition of immunoglobulin (IgM) and complement or its membrane attack complex has been reported on the surface of myelin sheaths (Dalakas et al. 1980). Antibodies may bind to macrophages via their Fc portion, activating phagocytosis and release of inflammatory mediators toward the myelin sheath. In addition passive transfer studies imply that these antibodies are important in pathogenesis (Yan et al. 2000). How cellular and humoral responses interact in CIDP is largely unknown. In addition little is known as to whether there is pathological heterogeneity in keeping with clinical heterogeneity.

#### 1.4.4. Diagnosis of CIDP

The EFNS taskforce agreed in 2005 on establishing a set of criteria for diagnosing CIDP (Joint Task Force of the EFNS and the P.N.S. 2005). Based on clinical, electrophysiological and supportive paraclinical information these guidelines allow the diagnosis of definite, probable and possible CIDP. The clinical picture is either “typical” or “atypical” as discussed in section 1.4.2. Amongst others exclusion criteria included antibodies to myelin-associated glycoprotein.

The electrophysiological features of CIDP are suggestive of demyelination and include reduced conduction velocities, temporal dispersion of the compound muscle action potential (CMAP) and conduction block (“Research criteria for diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP). Report from an Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force”, 1991) (Lewis et al. 1982) (Sumner 1994).

In accordance with reported neurophysiologic abnormalities definitive electrophysiological criteria have been defined and these include (Joint Task Force of the EFNS and the P.N.S. 2005):

1.  $\geq 50\%$  prolongation of distal motor latency in 2 nerves or
2.  $\geq 30\%$  reduction of motor conduction velocity in 2 nerves or
3.  $\geq 20\%$  prolongation of F-wave latency in 2 nerves or
4. Partial motor conduction block in 2 nerves ( $\geq 50\%$  amplitude reduction of proximal negative peak CMAP relative to the distal) or
5. Abnormal temporal dispersion in 2 nerves or
6. Absent F waves in 2 nerves + one other demyelinating parameter in another nerve (meeting criteria 1-6)
7. Prolonged CMAP duration ( $> 9\text{ms}$ ) in 1 nerve + one other demyelinating parameter in another nerve (meeting criteria 1-6)

Further criteria were established to allow neurophysiologic findings to be defined as probable (e.g. allowing partial conduction block to be defined when only  $\geq 30\%$  amplitude reduction of proximal negative peak CMAP relative to the distal) and possible CIDP. These neurophysiologic criteria must be interpreted in the setting of the clinical context as for example demyelination may be patchy or sometimes restricted to proximal nerves and roots therefore these strict criteria may not be met. Therefore the diagnosis is a clinical one supported by additional paraclinical data including the presence of raised protein in an acellular CSF, thickened or enhancing nerve roots on MRI on spinal imaging, a positive response to immunomodulatory therapy or unequivocal nerve biopsy with features consistent with demyelination and remyelination (Lunn et al. 2009).

CIDP share many symptoms and signs in early disease with GBS. A proportion of patients demonstrate an acute onset not unlike GBS - 16% in McCombe's

cohort with 35% giving a history of preceding infection(McCombe et al. 1987). Recent work by van Doorn et al suggest that the diagnosis of CIDP should be considered when a patient thought to have GBS deteriorates again after 8 weeks from onset or when deterioration occurs 3 times or more. The similarities between these two disorders have led some to speculate that they are variants of the one inflammatory neuropathy with the main difference between the two variants being the duration of symptoms(van Doorn 2005).

#### 1.4.5. Treatment of CIDP

A significant proportion of CIDP patients will respond to immunomodulatory therapies. First line therapies include steroids, intravenous immunoglobulin (IVIG) and plasma exchange (PE)(Lunn et al. 2009). Steroids were the first immunomodulatory treatment used in CIDP with case series suggesting that 65-95% respond(Austin 1958)(Dalakas et al. 1981)(McCombe et al. 1987)(Barohn et al. 1989).Side effects limit the use of steroids therefore the use of IVIG is preferable and therefore there is more evidenced based data available. The recent ICE study, a RCT investigating IVIG versus placebo in CIDP, reported an immediate response rate to IVIG was 54% vs. 21% in the placebo group. The absolute risk reduction for maintaining stability in prolonged treatment was 34%. The number needed to treat was 3.

Plasma exchange has been investigated in short term trials only but does show benefit(Dyck, Daube, O'Brien, Pineda, Low, Windebank & Swanson 1986b)(Hahn et al. 1996).IVIG has been compared with both steroids and PE with similar efficacies reported for all treatments(Hughes R. Bensa S. Willison H. Van den Bergh P. Comi G. Illa I. Nobile-Orazio E. van Doorn P. Dalakas M. Bojar M. Swan A. Inflammatory Neuropathy Cause and Treatment (INCAT) Group 2001)(Dyck et al. 1994). Numerous second line agents have been used with varying degrees of success including, amongst others, methotrexate, azathioprine, ciclosporin, cyclophosphamide and rituximab(Hughes RA. Raphaël JC. Swan AV. Doorn PA 2004). There is

however, as yet, no robust RCT evidence for treatment with these second line agents.

#### 1.4.6. Antiglycolipid antibodies and CIDP

The search for the specific antigenic target in CIDP has included investigation into both myelin derived proteins and lipids. However antibodies to P<sub>0</sub> and other myelin proteins such as PMP22 and P2 are reported with inconsistent frequencies in studies (Allen et al. 2005). However different frequencies may simply reflect differences in the conformation of the protein used in experimental protocols. P<sub>0</sub>, the most abundant peripheral nerve myelin protein, has been reported in 6/21 patients with CIDP responsive to PE and in 1/15 controls (Yan et al. 2001). P<sub>0</sub> appears to be the most likely protein antigenic target as it has been shown that antibodies directed against such proteins can lead to demyelination upon intraneural injection (Hughes et al. 1985).

Galactocerebroside, a major peripheral nerve glycolipid, is highly neurotoxic in animal models causing a CIDP-like process upon immunisation (Stoll et al. 1986) (Toyka et al. 1996). However antibodies against such lipid antigens have not been described in CIDP populations. The closely related lipid, sulfatide, has been investigated in numerous studies. Fredman et al reported that 87% (13/15) CIDP patients had detectable anti-sulfatide IgG using TLC (Fredman, Vedeler, Nyland, Aarli & Svennerholm 1991b). However this finding has not been reflected in other study findings (Ilyas, Mithen, Dalakas, Chen & Cook 1992a) (Melendez-Vasquez et al. 1997). Antibodies to a variety of lipid antigens have been described including LM1, GM1 and GD1a (Fredman, Vedeler, Nyland, Aarli & Svennerholm 1991a) (Ilyas, Mithen, Dalakas, Chen & Cook 1992a). Antibodies to GM1 are well described in the chronic immune neuropathy, MMN, however they present less frequently in CIDP (van Schaik, Bossuyt, Brand & Vermeulen 1995a). Human peripheral nerve myelin contains acidic glycosphingolipids such as sulfated glucuronyl paragloboside (SGPG) and SGLPG (sulfated glucuronyl lactosaminyl

paragloboside)(Quarles 1997)(Willison et al. 2002). One study found elevated IgM anti-SGPG in 6/9 CIDP patients(Yuki, Tagawa & Handa 1996a).

Table 1.1. Antiglycolipid antibody studies in CIDP.

Adapted from Hughes et al(Hughes et al. 2006).

Study	Antigen	Method	Class	CIDP positive	OND positive	HC positive
(Hughes et al. 1984)	GalC	ELISA	IgG	1/11	2/17 (GBS)	0/19
(Rostami et al. 1987)	GalC	RIA		No significant difference between 11 CIDP and 6 normal subjects		
(McCombe, Pollard & McLeod 1988b)	GalC	ELISA		0/57	0/49	
(Fredman, Vedeler, Nyland, Aarli & Svennerholm 1991b)	Sulf	TLC	IgG	13/15	15/23	6/40
(Ilyas et al. 1991)	Sulf	ELISA, TLC	IgM	1/15	11/53 (GBS)	
(Pestronk et al. 1991)	Sulf	ELISA, TLC		0/21	5/53	0/35
(Ilyas, Mithen, Dalakas, Chen & Cook 1992a)	Sulf	ELISA, TLC	IgG & IgM	0/16	18/64	0/35
(Melendez-Vasquez et al. 1997)	Sulf	ELISA	IgG	1/40	5/53 (GBS)	1/32
			IgM	0/40		0/37
(Quarles et al. 1990)	Gang	TLC		3/19	0/40	0/37
(Ilyas, Mithen, Dalakas, Chen & Cook 1992a)	GM1		IgM	2/16	5/26 (GBS)	0/10
(Simone et al. 1993)	GM1	ELISA, TLC	IgG(IgM)	1/10 (0/10)	9/23 (4/24)	1/55
(van Schaik et al. 1994)	GM1	ELISA	IgG	10/43	3/30	
			IgM	3/43	2/30	
(van Schaik, Bossuyt, Brand & Vermeulen 1995b)	GM1	ELISA		12/43		
(Yuki, Tagawa & Handa 1996a)	GM1		IgG	1/30	33/96 (GBS)	6/50
			IgM	5/30	35/96 (GBS)	3/50
(Melendez-Vasquez et al. 1997)	GM1	ELISA	IgG	0/40	0/40	0/37
			IgM	6/40	2/10	1/37
(Ilyas, Mithen, Dalakas, Chen & Cook 1992a)	LM1	ELISA & TLC	IgG	2/16	11/53 (GBS)	0/32
(Melendez-Vasquez et al. 1997)	LM1	ELISA	IgG	4/40	1/40	0/37
			IgM	2/40	4/40	1/40
(Ilyas et al. 1991)	SGPG	ELISA, TLC	IgM	1/15	13/53	
(Ilyas, Mithen, Dalakas, Chen & Cook 1992b)	SGPG	ELISA, TLC	IgM	0/16	7/53	0/32
(Yuki, Tagawa & Handa 1996b)	SGPG	ELISA	IgG	1/30	0/96	0/50
			IgM	12/30	28/96	5/50



Key:

GalC= Galactocerebroside

Sulf= Sulfatide

Gang= Human gangliosides

## 1.5 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic disease of the central nervous system (CNS) in which repeated episodes of inflammation result in extensive demyelination, axonal loss and chronic disability and was first described over 150 years ago. It is the most common non traumatic disabling neurological condition affecting young adults and affects over one million people aged from 17 to 65 years worldwide (Anderson et al. 1992). The estimated prevalence of MS in Europe is 83 per 100,000, with higher rates observed in northern countries such as Scotland (Pugliatti et al. 2006).

### 1.5.1. Epidemiology of Multiple Sclerosis

Development of MS depends on both genetic susceptibility and environmental factors (Poser 2006) (Poser et al. 2004). How these complex factors interact is largely unknown. The importance of genetic factors is supported by the familial aggregation of disease (Table 1.2), the high prevalence in specific ethnic populations and the absence of increased risk of MS in adopted relatives of those with MS.

Table 1.2. The genetics of Multiple Sclerosis (Poser 2000) (Poser et al. 2004)

Relative with MS	Risk of developing MS (%)	Increase in risk above general population *
Monozygotic twin	25-30	125-150x
1 <sup>st</sup> degree relative	2-4	10-20x
Offspring of conjugal mating	30.5	150x

\* Risk of developing MS in general population is 0.2%

\*\* Both parents have MS

The influence of environmental factors on MS risk is suggested by a variation in disease incidence with a higher incidence in temperate climates further away from the equator. Epidemiological data from migrant populations suggest early life environment is important. Those emigrating before the age of 15 from a high risk zone to a low risk zone carry at least part of the high risk of their country of origin (Poser et al. 2004).

### 1.5.2. Clinical features of Multiple Sclerosis

A clinically heterogeneous disease, MS is classified based on the clinical presentation at onset (Lublin et al. 1996) (Polman et al. 2005). A clinically isolated syndrome (CIS), caused by an inflammatory lesion involving the white matter of the central nervous system, is associated with risk of progression to clinically definite MS. The most common MS subtype, relapsing remitting MS (RRMS), is diagnosed when there have been two or more attacks of CNS demyelination lasting over 24 hours, separated by one month or more (i.e. relapses). Relapses last for a number of days or weeks. Resolution of symptoms after a relapse may be a result of diminution of inflammation or remyelination. After 20 years around 80% of RRMS patients will develop progressive neurological decline with acute relapses of neurological decline becoming less evident. Primary progressive MS (PPMS) occurs in an older population and presents with progressive neurological decline.

### 1.5.3. Pathology of Multiple Sclerosis

The pathology of MS has similarly been shown to be heterogeneous. Historically the pathological basis of MS has been considered to be T cell dependent with inflammatory process instigated by myelin reactive T cells leading to secondary macrophage recruitment and myelin destruction (Lassmann et al. 2001) (Pittock et al. 2007). This has been illustrated by the induction of the animal form of MS, experimental autoimmune encephalitis (EAE), after the adoptive transfer of CD4<sup>+</sup> T cells. However increasing evidence indicates B cells also contribute to disease pathogenesis (Lucchinetti et al. 2000) (Hauser et al. 2008). Indeed both cells of the innate immune system (macrophages and microglia) and the adaptive immune system (T cells, B cells and plasma cells) can be identified in active MS lesions (Lucchinetti et al. 2000).

Recent neuropathological studies revealed that there are four patterns of demyelination observed amongst active MS lesions obtained via brain biopsy and at time of autopsy (Lucchinetti et al. 2000) (Lassmann et al. 2001). A T cell dominant inflammatory process was observed in all four patterns however the investigators were able to segregate the lesions into four distinct groups based on plaque geography, extent and pattern of oligodendrocyte pathology, evidence for immunoglobulin deposition and complement activation, and pattern of myelin protein loss (Table 1.5.). Interestingly only one pathological pattern was observed per patient. The most common pattern observed, Type II, was characterized by the presence of complement activation and Ig deposition at sites of active myelin damage, suggesting that these mechanisms may be involved in demyelination and tissue injury. The efficacy with which plasma exchange was observed at treating pattern II patients supports this hypothesis (Keegan et al. 2005). In addition a recent phase II double blinded placebo controlled trial investigating the B cell depleting agent Rituximab in RRMS would appear to support the suggestion that B cells have a pathogenic role in

MS(Hauser et al. 2008)(Lassmann et al. 2001). The trial revealed promising results, with a reduction in total (and new) gadolinium enhancing lesions and a reduction in relapses (20.3% vs. 40%) observed in the treated group.

**Table 1.3. Pathological heterogeneity in multiple sclerosis**  
(Adapted from the Pathology of MS(Pittock et al. 2007))

Pathology subtype	Frequency (%)	Pattern of demyelination
I	15	Macrophage associated demyelination
II	58	Antibody and complement associated demyelination
III	26	Distal oligodendrocyte dystrophy
IV	1	Primary oligodendrocyte injury in periplaque white matter with secondary macrophage associated demyelination

### 1.5.3. Diagnosis of Multiple Sclerosis

A diagnostic scheme, employing data from paraclinical parameters such as Magnetic Resonance Imaging (MRI), is commonly used to reliably diagnose MS(Polman et al. 2005) (Table 1.3. and Table 1.4.)

Table 1.4. Diagnosis of clinical subtypes of multiple sclerosis.

(Adapted from the “McDonald Criteria”) (Polman et al. 2005)

Clinical Presentation	Additional data required
PPMS	One year of disease progression Plus 2 of the following: 1. Positive MRI Brain (nine T2 lesions or four or more T2 lesions with positive visual evoked potentials (VEP) <sup>a</sup> ) 2. Positive spinal cord MRI (two focal T2 lesions) 3. Positive cerebrospinal fluid (CSF) <sup>b</sup>
Two or more attacks; objective clinical evidence of two or more lesions	None
Two or more attacks; objective clinical evidence of one or more lesion	Dissemination in time, demonstrated by: <i>MRI<sup>c</sup> or</i> Two or more MRI-detected lesions consistent with MS plus positive CSF <i>or</i> Await further clinical attack implicating a different site
One attack; objective clinical evidence of two or more lesions	Dissemination in time, demonstrated by: <i>MRI<sup>c</sup> or</i> Second clinical attack
One attack; objective clinical evidence of one lesion (clinically isolated syndrome)	Dissemination in space, demonstrated by: <i>MRI<sup>d</sup> or</i> Two or more MRI detected lesions consistent with MS plus positive CSF <i>and</i> Dissemination in time, demonstrated by: <i>MRI or</i> Second clinical attack

<sup>a</sup> Abnormal VEP of the type seen in MS<sup>b</sup> Positive CSF by conventional methods such as isoelectric focusing of oligoclonal IgG bands or increased IgG index or both<sup>c</sup> MRI dissemination in time must fulfil the criteria in Table 5.3.<sup>d</sup> MRI dissemination in time must fulfil the criteria in Table 5.3.

Table 1.5. Magnetic resonance imaging criteria to demonstrate dissemination of lesions in time and space.

(adapted from “McDonald Criteria”(Polman et al. 2005))

MRI parameters	Diagnostic criteria
Dissemination in time	Detection of gadolinium enhancement at least 3 months after the onset of the initial clinical event Or Detection of a new T2 lesion occurring 30 days after reference scan
Dissemination in space	3 of the following: 1. One gadolinium enhancing lesion or nine T2 lesions 2. One juxtacortical lesion 3. One infratentorial lesion 4. Three periventricular lesions

Symptoms attributable to MS are generally indicative of damage to CNS white matter and can include painful loss of vision secondary to optic neuritis, coordination problems with cerebellar disease and leg weakness secondary to myelitis. Relapses may be monofocal or multifocal. Multifocal episodes with little recovery between are predictive of a more severe outcome(Langer-Gould et al. 2006).

### 1.5.5. Treatment of Multiple Sclerosis

MS treatment consists of three modes of treatment:

1. Treatment of acute relapses with intravenous steroids or plasma exchange
2. The use of disease modifying therapies
3. Symptomatic treatments

In contrast to the use of immunosuppressants (usually steroids) for acute relapses, the use of disease modifying therapies in MS influence disease activity. Glatiramer Acetate and a number of interferon  $\beta$  (IFN  $\beta$ ) preparations have been shown to improve the course of the disease by reducing the relapse rate by around 30%(Comi et al. 2001)(Río J. Tintoré M. Nos C. Téllez N. Galán I. Montalban X 2005). In addition these therapies have been shown to reduce MRI parameters for disease activity and severity.

A number of emerging agents have evolved in recent years including many oral agents and monoclonal antibody therapies. Many of these have diverse mechanisms of action. A number of these target T cell dependent responses for example Natalizumab, which was the first targeted therapy used in MS. This recombinant humanized antibody targets an adhesion molecule,  $\alpha 4\beta 1$ -integrin, on the surface of T cells. This interaction prevents T cells attaching to endothelial cells via vascular cell adhesion molecule-1 (VCAM-1) and thereby halts the traffic of T cells across the blood brain barrier and is a highly effective treatment in MS(Ransohoff 2007). However, increasing evidence is gathering for the role of B cell ablative therapy in MS. Rituximab, the chimeric murine/human monoclonal antibody which depletes



B cells which express the target antigen CD20, is the B cell antagonist of interest. This treatment effectively depletes B cells from CSF and blood, lysing circulating B cells whilst sparing stem cells and mature plasma cells. Recent work in RRMS has demonstrated that Rituximab significantly reduces MS lesions and clinical relapses over a period of 48 weeks compared with placebo (Hauser et al. 2008). This adds further credence to the role of the humoral immune response in the pathogenesis of Multiple Sclerosis.

#### 1.5.6. Oligoclonal bands

Indeed the most obvious indication that MS is associated with an aberrant humoral response is an increased intrathecal synthesis of IgG that manifests itself as discrete oligoclonal bands (OCB) of IgG in cerebrospinal fluid only (see Figure 5.1. (A) ) (Correale et al. 2002). OCB are immunoglobulins (IgG, IgM or IgA) that are generated by plasmablasts and plasma cells in the CSF or CNS compartment. Isoelectric focusing (IEF) allows the separation of proteins in biological fluids according to the isoelectric point. They are visualized as prominent distinct bands in polyacrylamide gels. An illustrative IEF blot demonstrating the common patterns observed in clinical practice is shown in Figure 1.4.

OCB are the most important diagnostic laboratory marker in practice in multiple sclerosis (Polman et al. 2005). In CIS the presence of OCB has been shown to be a valuable predictive biomarker. One particular prospective study following over 400 patients with a diagnosis of CIS, demonstrated that the presence of OCB doubled the risk for having a second demyelinating attack within 5 years (Tintore et al. 2008).

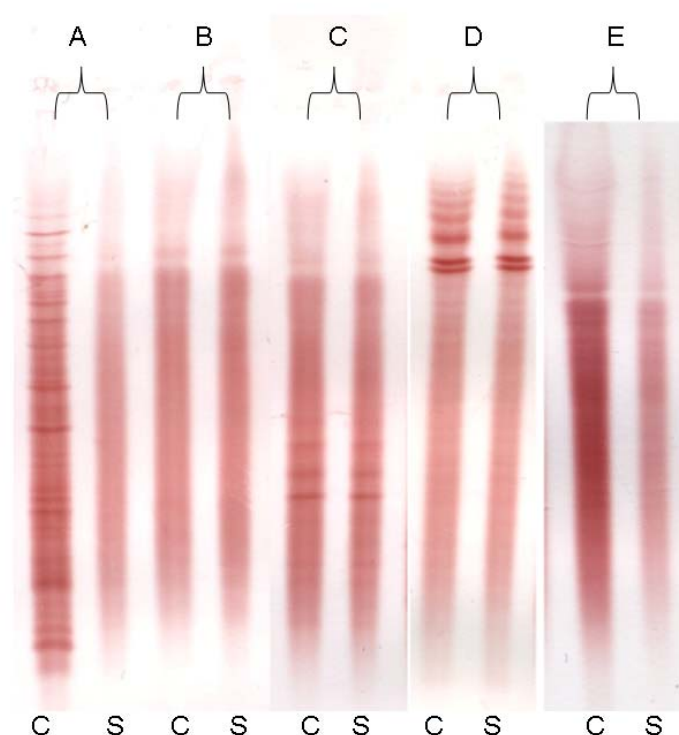


Figure 1.4. IEF Blot of CSF proteins and corresponding serum

(courtesy of Ms Patricia Thomson & Ms Jan Gairns, NHS Neuroimmunology Laboratory, Southern General Hospital, Glasgow)

A. Oligoclonal bands detected in the CSF only, and not in serum, indicative of local synthesis of immunoglobulin within the CNS compartment (typical pattern seen in MS)

B. Normal examination

C. Paired oligoclonal bands detected in serum and CSF indicative of recent antigenic stimulation outside the CNS e.g. as aftermath of systemic infection

D. Monoclonal paraprotein detected in the serum also present in the CSF (characteristically seen as a consequence of a monoclonal M protein in multiple myeloma or monoclonal gammopathy of uncertain significance)

E. Polyclonal increase in CSF gamma globulin

OCB are derived from clonally expanded populations of B cells that have undergone numerous somatic hypermutations indicative of antigen-specific selection, but their specificity and pathobiological relevance remains unknown(Qin et al. 1998)(Baranzini et al. 1999)(Ritchie et al. 2004). Despite intensive research, the target antigen(s) recognized by individual OCB in MS has remained elusive(Meinl et al. 2006)(Awad et al. 2010).

#### 1.5.7. Anti glycolipid antibodies and MS

Much attention to date has focused on potential immune responses to myelin-derived proteins in the hunt for the OCB target antigens. This search for protein derived targets of the OCB response has, as yet, proven unfruitful(Owens et al. 2009). However 70-85% of the dry weight of myelin is composed of lipids whilst the remainder is comprised of proteins such as myelin basic protein and myelin oligodendrocyte protein(Norton 1977). Glycolipids such as gangliosides and galactocerebrosides are the most abundant component of human myelin. Cerebrosides are a subtype of glycosphingolipid in which the ceramide has a sugar residue at 1-hydroxy residue. The sugar residue can be either glucose or galactose; the two major subtypes are glucocerebroside and galactocerebroside. Galactocerebroside is one of the simplest glycolipid structures consisting of a galactose molecule linked to a ceramide. Sulfatide, another predominant myelin lipid is a galactocerebroside in which the 3'OH moiety on galactose is sulfated. (Figure 1.5.) Cerebrosides are the major glycolipid in CNS myelin(Norton 1977).

Both serum and CSF anti-ganglioside antibodies have been reported in multiple sclerosis patients(Arnon et al. 1980)(Acarin et al. 1996, Zaprianova et al. 2004)(Kanter et al. 2006). In fact 25 years ago it was observed that antisera to different glycolipids induced myelin alterations in mouse spinal cord tissue cultures(Roth et al. 1985). Well myelinated cultures of mouse spinal cord tissue were exposed to antisera against GalC, GM1 and GM4 and

anti-white matter serum. Cultures exposed to anti-white matter serum and anti-Galactocerebroside (GalC). antisera showed the most marked changes with a decrease in the number of oligodendroglial cells with dissolution and phagocytosis of myelin evident (assessed using light and electron microscopy). With higher concentrations of antisera, antibodies to GM1 and GM4 demonstrated similar albeit less severe changes. This difference in demyelinating capabilities of the antisera may be related to the amounts of respective antigen determinants present in the myelin membrane with GalC accounting for 24% of total myelin lipids whilst gangliosides account for less than 1% of this fraction (Norton 1977, Shamshiev et al. 1999). The authors postulate that the damage induced correlated with the amount of lipid target present in the membranes involved.

Glycolipids themselves are potential targets recognizable by T cells in MS patients. T cell clones reactive to single glycolipids have been isolated from the peripheral blood of MS patients (Shamshiev et al. 1999). Some clones (3/21) were found to react only to glycolipids when presented in a mixture with other glycolipids (40% gangliosides, <25% sphingomyelin, <15% ceramides, <6% sulfatide and <15% N-acetylneuraminic acid). This raises the possibility that glycolipids may interact with each other forming new structures i.e. complexes with new epitopes capable of stimulating T cells which were otherwise unreactive when presented to isolated gangliosides.

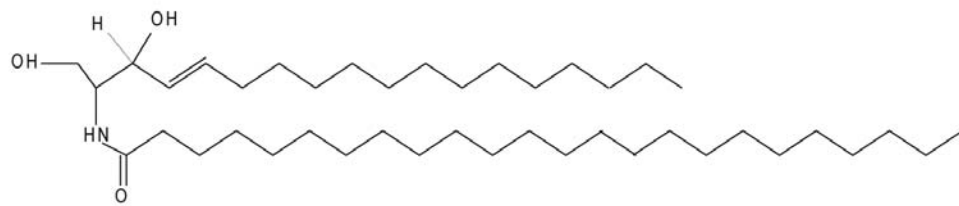
Anti-sulfatide antibodies have previously been reported in CSF of MS patients (Ilyas et al. 2003) (Kanter et al. 2006). Sulfatides, a class of sulfated galactoceramides, are synthesized primarily by the oligodendrocytes of the central nervous system. Although sulfatide is present in most cell membranes it is particularly enriched in the myelin sheath, which is the target of inflammation in multiple sclerosis. Sulfatide has been shown to be the most promiscuous of all lipids as it is capable of binding to all CD1 isotypes whereas other lipids are isoform specific (Shamshiev et al. 2002). CD1 molecules are MHC class 1 like molecules that present lipid antigens to

Natural Killer T cells. The CD1 family consists of 5 glycosylated proteins, CD1a, CD1b, CD1c, CD1d and CD1e, which have limited polymorphism and are all expressed by professional antigen presenting cells (Porcelli 1995).

Recently it has been shown that sulfatide reactive CD1d T cells represent a distinct population of T cells that infiltrate the CNS during experimental autoimmune encephalitis (EAE) (Jahng et al. 2004). Disease suppression was possible in the animal model when the animals were treated at the same time as being challenged with MOG 35-55/CFA/PT for the induction of EAE. Suppression of disease was associated with a suppression of interferon- $\gamma$  and interleukin-4 production by pathogenic myelin oligodendrocyte glycoprotein-reactive T cells. Co injection with other self-glycolipids including GM1, sphingomyelin or  $\beta$  Galactoceramide had no effect on the course of EAE.

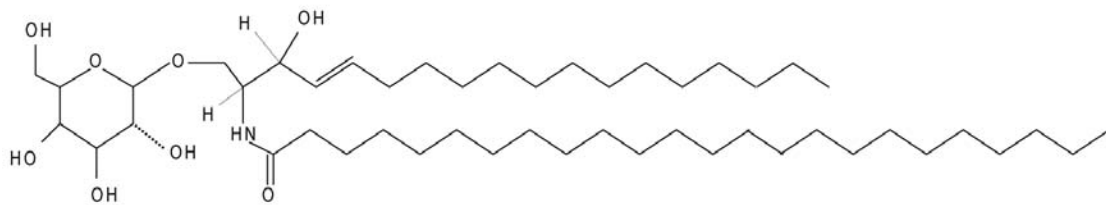
The recently described microarrays have demonstrated the presence of antibodies in the CSF directed against a variety of lipid antigens including sulfatide (Kanter et al. 2006). 50 different lipids were sprayed onto PVDF membranes which were then incubated with CSF obtained from patients with MS (n=16) and patients with other neurological disease (n=11). The MS samples clustered showing strong IgG reactivity to lipids including sulfatide, a variety of oxidized lipids, phosphatidylethanolamine, sphingomyelin and lysophosphatidyl ethanolamine. In the SPMS group (n=8) antibodies were detected against GM1 and asialo GM1. The same group also reported that antibodies directed against myelin lipids (sulfatide, cerebroside, asialo GM1) appeared in mice after EAE had been induced. Administering a dose of sulfatide, as opposed to cerebroside, along with PLP (the antigen used to induce EAE) lead to a much worse clinical outcome. In addition administration of sulfatide-specific antibody worsened the disease severity in EAE.

A



B

Ceramide galactosyltransferase



Sulfotransferase

C

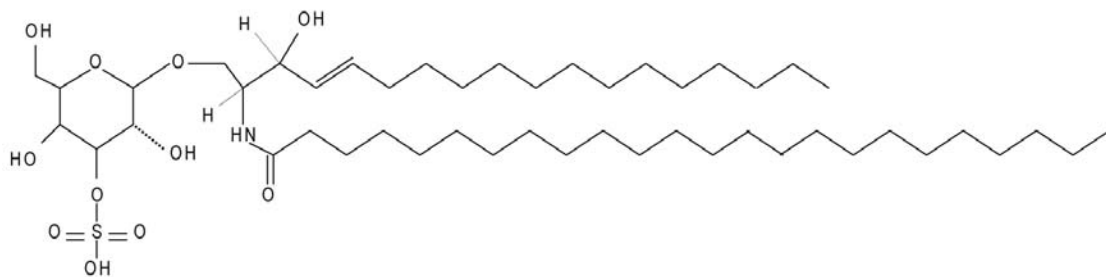


Figure 1.5. Molecular structure of galactolipids

A= Ceramide

B= Galactocerebroside

C= Sulfatide

The deleterious effect of sulfatide on EAE has also been demonstrated in guinea pigs with increased demyelination evident (Moore et al. 1984) .

Furthermore the implantation of a hybridoma secreting sulfatide-specific antibody into the spinal cord of rats has been shown to cause demyelination (Rosenbluth et al. 2003). It is not clear why the T cell study has conflicting results to Kanter et al. However not only do the times and doses of sulfatide used in both studies differ but also the animal models and myelin antigen used to induce EAE.

This work is supported by an earlier study by Ilyas et al who investigated IgG antibodies directed against glycolipids of CSF in MS patients and controls (Ilyas et al. 2003). They reported that CSF antisulfatide antibodies were detectable in all subtypes of MS with a higher frequency of antibodies in SPMS (30%) compared to RRMS (15%) and PPMS (14%). The purpose of such antisulfatide antibodies is yet to be determined however a sulfatide reactive monoclonal IgM antibody has been isolated from a patient with MS and shown to bind selectively to living oligodendrocytes in brain cell cultures of newborn rats (Rosenbluth et al. 2003). Staining of living cultures revealed that the monoclonal antibody bound to cells with typical oligodendrocyte morphology i.e. with round cell bodies, multiple arborized processes and large elaborated membrane sheets. The role of this sulfatide reactive antibody was not elucidated by this study. The lipid sulfatide, a product of oligodendrocytes, is first produced at a critical stage of oligodendrocyte differentiation. The authors postulate that these antibodies, capable of binding to the oligodendrocyte, interfere with oligodendrocyte differentiation thereby inhibiting repair. Equally these antibodies may be responsible for the prevention of recognition of exposed sulfatide by CD1 restricted natural killer T cells. Thus by preventing the presentation of such lipid antigens to T cells they may act to inhibit the host immune response.

## 1.6. Summary:

The influence of neighbouring glycolipids on antibody binding is an intriguing concept with potential repercussions in a host of neurological diseases. This concept may explain the inconsistencies observed between the anti-ganglioside profile and clinical phenotype in GBS. The axonal phenotypes AMAN and AMSAN are distinct clinical phenotypes yet both are associated with antibody directed against the same antigen, GM1, which is present in equal amounts in motor and sensory nerves. One postulation may be that in AMAN cases, the GM1 ganglioside in sensory nerves is “shielded” from antibody binding by adjacent “clustered” gangliosides.

The clustering of ganglioside epitopes preventing the binding of IgG may also explain the finding of undamaged nerves with high GQ1b content in Miller Fisher Syndrome (Chiba et al. 1997). In addition the lack of a well defined antigenic target associated with the demyelinating phenotype, AIDP, is well described. One may hypothesize that the search for antigenic targets in AIDP has been unrewarding to date because of the lack of understanding of the importance of the neighbouring glycolipid environment.

My hypothesis is that the creation of an array based platform will lead to the identification of additional antigenic targets in the peripheral neuropathies GBS and CIDP. This may include the identification of anti-ganglioside complex antibodies which may help stratify patient groups according to clinical phenotype. In addition I hypothesize that this technique may allow us to investigate further the specificity of CSF immunoglobulin in Multiple Sclerosis patients.

To further understand the influence of the local environment on the binding capabilities of immunoglobulin it is necessary to design a new platform to



provide screening of a high number of antigens. Standard ELISA techniques are limited not only in the number of antigens that can be assayed but also by the amount of antigen and antibody required. An array based method would potentially allow a higher number of antigens to be investigated.

The aims of this thesis were therefore to:

1. Develop a “glycoarray” protocol to allow analysis of a large number of potential glycolipid antigens
2. Compare the new protocol to standardized well established techniques such as ELISA
3. Characterize anti-ganglioside complex antibodies in Guillain Barré Syndrome (GBS)
4. Characterize anti-ganglioside complex antibodies in Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)
5. Characterize anti-myelin lipid complex antibodies in Multiple Sclerosis (MS)
6. Attempt to further develop and miniaturize the glycoarray protocol to allow analysis of both protein and glycolipid antigens and complexes

## Chapter 2. Methods

### 2.1 ELISA

After sonication for 3 minutes, 100ul of ganglioside (all lipids used in experiments are detailed in Table 2.1) in methanol solution at a concentration of 2ug/ml was placed onto 96-well Immulon 2 HB microtitre plates (Dynatech, Billingham, UK). Methanol was then evaporated leaving a final concentration of 200ng per individual well. After evaporation plates were placed at 4°C for a minimum of one hour. Non-specific binding to the plates was blocked by an hour long incubation at 4°C with 150ul per well of 2% fatty acid free bovine serum albumin (FAFBSA) in PBS (Europa Bioproducts Ltd, UK). BSA was then discarded and 100ul per well of diluted serum or monoclonal antibody was added. Serum was diluted in 0.1% FAFBSA. Plates were then incubated at 4°C. After 3 hours plates were then washed 6 times with PBS. 100ul of horseradish peroxidase (HRP) conjugated polyclonal rabbit anti-human IgG (DakoCytomation, Denmark) was then added (diluted 1:3000 in 0.1% FAFBSA) to each well. After incubation for 1 hour at 4°C plates were washed as before. Plates were then developed for 15 minutes in the dark at room temperature with 100ul per well of substrate solution (-an O-phenylenediamine tablet dissolved in a solution of 14ml 0.1M citric acid, 16ml 0.2M Na<sub>2</sub>HPO<sub>4</sub>, 30ml distilled water, 20ul H<sub>2</sub>O<sub>2</sub>). The reaction was then stopped with 50ul/well of 4M sulphuric acid. The optical density at 410nm was then determined. The background reading from a methanol coated well was subtracted from the ganglioside coated well to give an accurate reading. Coefficients of variation for this method have been reported as 11.2% for IgM and 3.8% for IgG glycolipid antibodies (Kuijff et al. 2005).

Table 2.1. Lipids used in ELISA and blot experiments

Lipid	Company	Source of lipid
GA1	Sigma-Aldrich	Bovine Brain
GM1	Sigma-Aldrich	Bovine Brain
GM2	Sigma-Aldrich	Bovine Brain
GM3	Sigma-Aldrich	Canine blood
GM4	Calbiochem	Human brain
GD2	Calbiochem	Human brain
GD3	Matreya	Bovine buttermilk
GD1a	Sigma-Aldrich	Bovine Brain
GD1b	Sigma-Aldrich	Bovine Brain
GT1a	Accurate Chemical &Scientific Corporation	Human brain
GT1b	Matreya	Bovine
GQ1b	Accurate Chemical &Scientific Corporation	Bovine brain
Ceramide (Cer)	Sigma-Aldrich	Bovine Brain
Cardiolipin (Cardio)	Sigma-Aldrich	Bovine Heart
Sulfatide (Sulf)	Sigma-Aldrich	Bovine Brain
Galactocerebroside (GalC)	Sigma-Aldrich	Bovine Brain
L alphaphosphatidylcholine (PC)	Avanti Polar Lipids	Brain, porcine
L alphaphosphatidylserine (PS)	Avanti Polar Lipids	Brain, porcine
L alphaphosphatidylethanolamine (PE*)	Avanti Polar Lipids	Brain, porcine
L alpha Phosphatidylinositol-4-phosphate (PIP (4))	Avanti Polar Lipids	Brain, porcine
Digalactosyl diglyceride (DGG)	Sigma-Aldrich	wholewheat
Galactosyl diglyceride (MGG)	Sigma-Aldrich	wholewheat
PC /Plasmalogen (PL)	Avanti Polar Lipids	Brain, porcine
Sphingosine (SS)	Sigma-Aldrich	Bovine brain
Sphingomyelin (SM)	Sigma-Aldrich	Bovine Brain
Cholesterol (Chol)	Sigma-Aldrich	Sheep's wool

## 2.2. Development of glycoarray methodology

### 2.2.1 The ECL method

Initially we employed the method described by Kanter et al (Kanter et al. 2006). Importantly this method investigated CSF and monoclonal antibody binding only and not serum. 0.2µm pore size polyvinylidene difluoride (PVDF) (Invitrogen, UK) membranes were cut (into equal sizes) and affixed using double sided Scotch tape (3M, USA) to plain glass microscope slides (VWR International, UK). A hydrophobic marker was then used to outline the border of the membrane. Lipid antigens of interest were dissolved from stock solutions to working solutions in methanol of 0.1mg/mL. Combinations of lipids were created in 50:50 mixture and stored in sealed rubber topped vials (Chromacol, UK). All lipids were sonicated for 3 minutes prior to application to the slides (Ultrawave Ltd, UK).

The experimental protocol echoes that of the ganglioside ELISA. The hydrophobic ceramide tail anchors the glycolipid to the hydrophobic membrane exposing the carbohydrate moiety to potential antibody binding. The experimental outline is summarised in Figure 2.1.

Using an ATS 4 machine (Camag), lipids were sprayed onto the membrane covered slides. The lipids were sprayed onto predetermined coordinates using the ATS 4 Freemove software. Slides were then left to dry for 1 hour. Slides were then placed on trays and probed with 2% fatty acid free bovine serum albumin (FAF BSA) in PBS for overnight at 4°C, to eliminate non-specific binding to the slides. BSA was then discarded and 500ul per slide of diluted serum or monoclonal antibody was added. Primary (serum or monoclonal) antibody was diluted in 0.1% FAF BSA/PBS. Slides were incubated at 4°C (on shaker at 4°C) for 2 hours. After 1 hour slides were then washed. This entailed incubating the slides in a jar/rack with PBS on a shaker (75rpm) for 2 hours at 4°C. The solution was refreshed every 30 minutes. Secondary antibody (polyclonal rabbit anti-human IgG (DakoCytomation, Denmark)) was then diluted in 0.1% FAF BSA/PBS and

slides were incubated at 4 °C (on shaker at 45rpm) for 2 hours. After incubation with the secondary antibody, a further series of washes was undertaken. After a final wash in distilled H<sub>2</sub>O, ECL solution (Amersham ECL Plus, GE Healthcare, UK) was applied to each slide for 5 minutes. Thereafter, slides were placed in a cassette (Kodak,UK). Trimmed transparency paper was placed overlying the slides in the cassette. Film was then placed over the slides in the dark room and exposures of varying lengths of time were obtained. Films were digitalized using a flatbed scanner (Epson DX 6000). TIFFs were then quantified using appropriate analytical software (Total Lab TL100, UK) which measures the pixel intensity of individual spots.

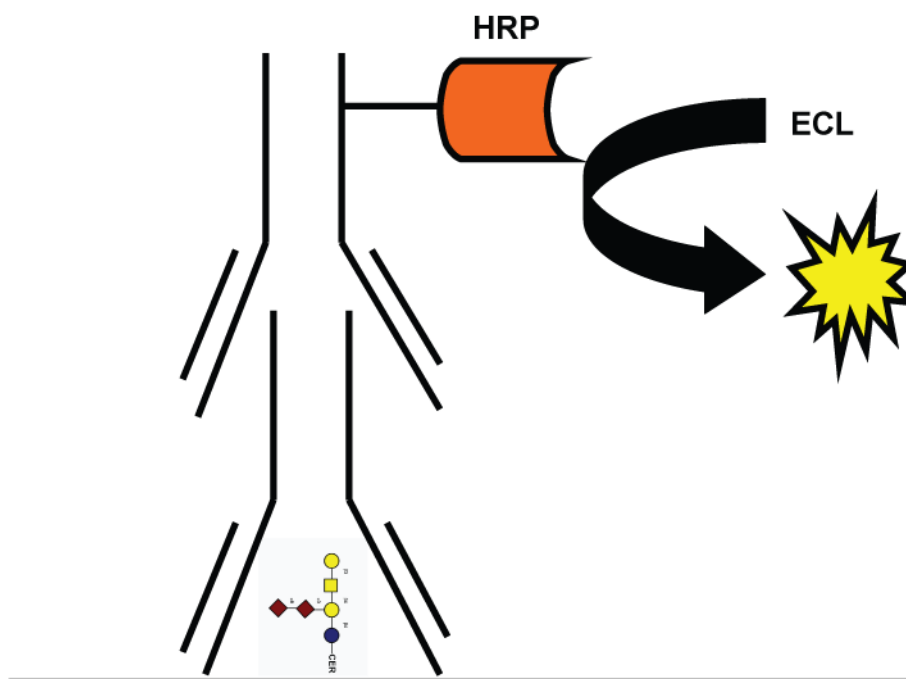


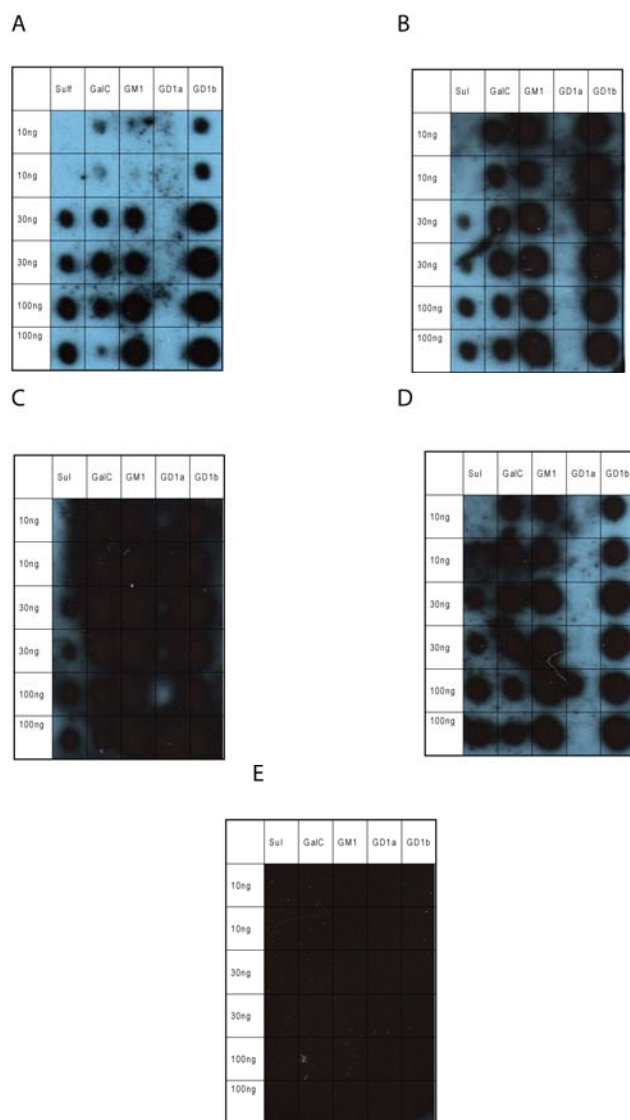
Figure 2.1. Experimental outline of combinatorial array

Lipid antigen (GD1b illustrated here) adheres to hydrophobic membrane exposing carbohydrate moiety. Primary antibody e.g. human GBS serum binds to lipid. Secondary anti- human IgG (which is linked to HRP) binds to the primary antibody. After application of ECL substrate a chemiluminescent reaction occurs which is then rendered on radiographic film.

## 2.2.2 Optimisation of the ECL method

### 2.2.2.1 Blocking experiments

Upon probing the membranes with diluted human serum it became evident that there were a proportion of samples with a high level of non specific binding to the PVDF membrane. A series of experiments investigating several different blocking methods was undertaken (Figure 2.2). All samples were assayed using 0.01% diluted human serum and 1/5000 anti-Human HRP-IgG. FAF BSA appeared to be of equivalent blocking to regular BSA (Sigma, UK) (data not shown). However other methods including commercial derived methods (e.g. Superblock (Thermo Scientific, Rockford, IL)) did not appear to be of superior efficacy in reducing the level of background binding to the PVDF membrane.



**Figure 2.2. Blocking experiment**

Shown are blots obtained for one serum, 070500, (a patient with GBS and known anti-GM1 and GD1b antibodies when tested using ELISA) using five different blocking methods. The lipids were sprayed in escalating amounts of 10ng, 30ng and 100ng.

A. Incubation of 1 hour in 2% FAF BSA

B. Incubation of 16 hours in 2% FAF BSA

C. Incubation of 1 hour in 5% FAF BSA

D. Incubated of hour in 2% non fat milk (in PBS) (Tesco, UK)

E. Incubated of 15 minutes in Superblock



#### 2.2.2.2. Detergent

We next investigated the addition of detergent to the wash cycles to see if this would reduce non specific binding to the PVDF membrane. Tween 20 (Roche, Germany) is a polysorbate surfactant which is relatively non-toxic and used commonly as a detergent in laboratory assays. We used Tween at concentrations of 0.001%, 0.01% and 0.05% (dissolved in PBS) as the primary wash solution in successive experiments and compared to using plain PBS as the primary wash solution.

Tween at a concentration of 0.001% was no different from PBS (data not shown) at reducing background binding. However even at a concentration of 0.01% there was an observed reduction in binding of human antibody to lipid antigen (Figure 2.3). Also the addition of detergent to the wash cycle reduced the adherence of the PVDF membrane to the glass slide and membranes commonly floated off the slides. Therefore the addition of detergent to wash cycles was abandoned.

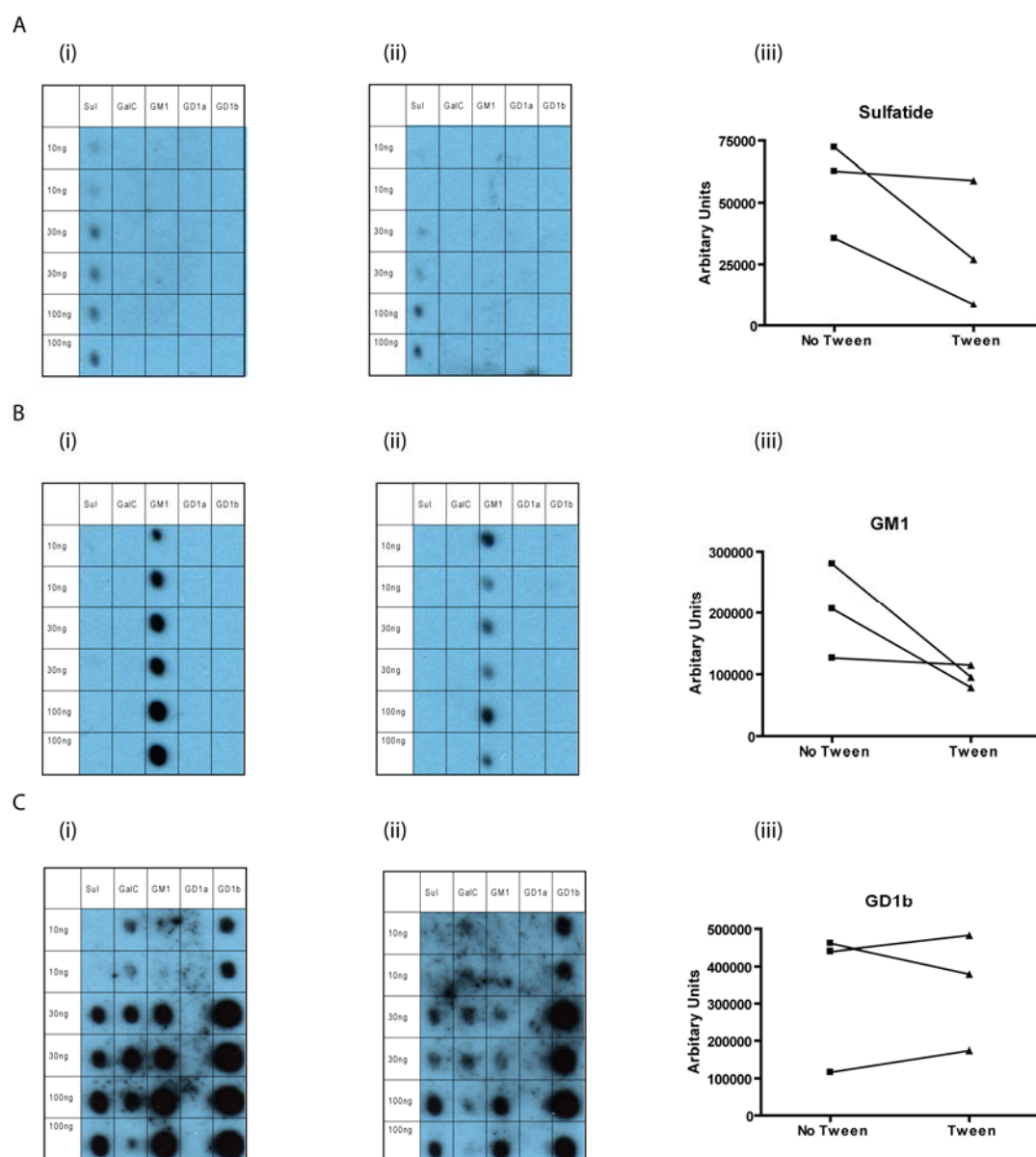


Figure 2.3. The addition of detergent to experimental protocol (Tween 0.01%).

Blots and quantitative data obtained from three human sera with different anti glycolipid antibody profiles. Arbitrary units of intensity were measured using Image Quant Total Lab100 software and were averaged for each of the concentrations of lipids spotted; 10ng, 30ng and 100ng.

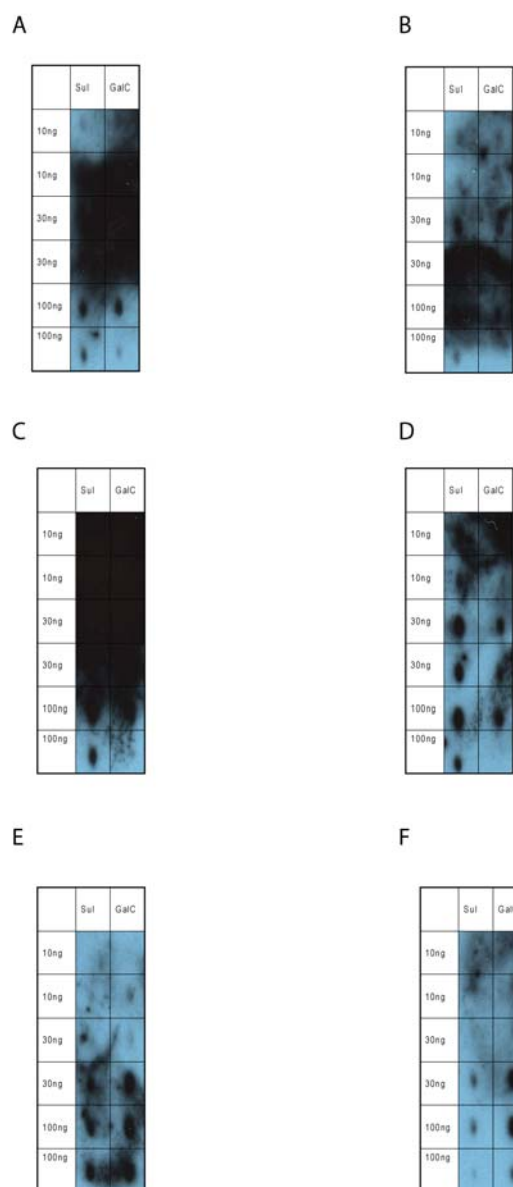
A. Anti sulfatide antibody (i) without Tween (ii) with Tween

B Anti-GM1 antibody (i) without Tween (ii) with Tween

C Anti GD1b antibody (i) without Tween (ii) with Tween

### 2.2.2.3 Preabsorption with PVDF

In an effort to try to reduce non specific background binding diluted serum samples, noted to have high background on previous experiments, were preincubated with blank PVDF membranes (Figure 2.4). All sera, with reactivity to sulfatide, were diluted to 0.01% in 0.01% FAF BSA. Each was then incubated with blank unused PVDF membrane in an eppendorf tube for 1 hour prior to the initial “blocking step”. Unfortunately this did not have a consistent diminutive effect on the level on background binding (Figure 2.4)



**Figure 2.4. Preabsorption with PVDF membrane**

Three human sera with known anti-sulfatide antibodies were assayed in duplication, with and without an initial preincubation with blank unused PVDF membrane.

A. Serum 43046

B. Serum 43046 assayed with initial preabsorption step

C. Serum 50635

D. Serum 50635 assayed with initial preabsorption step

E. Serum 64841

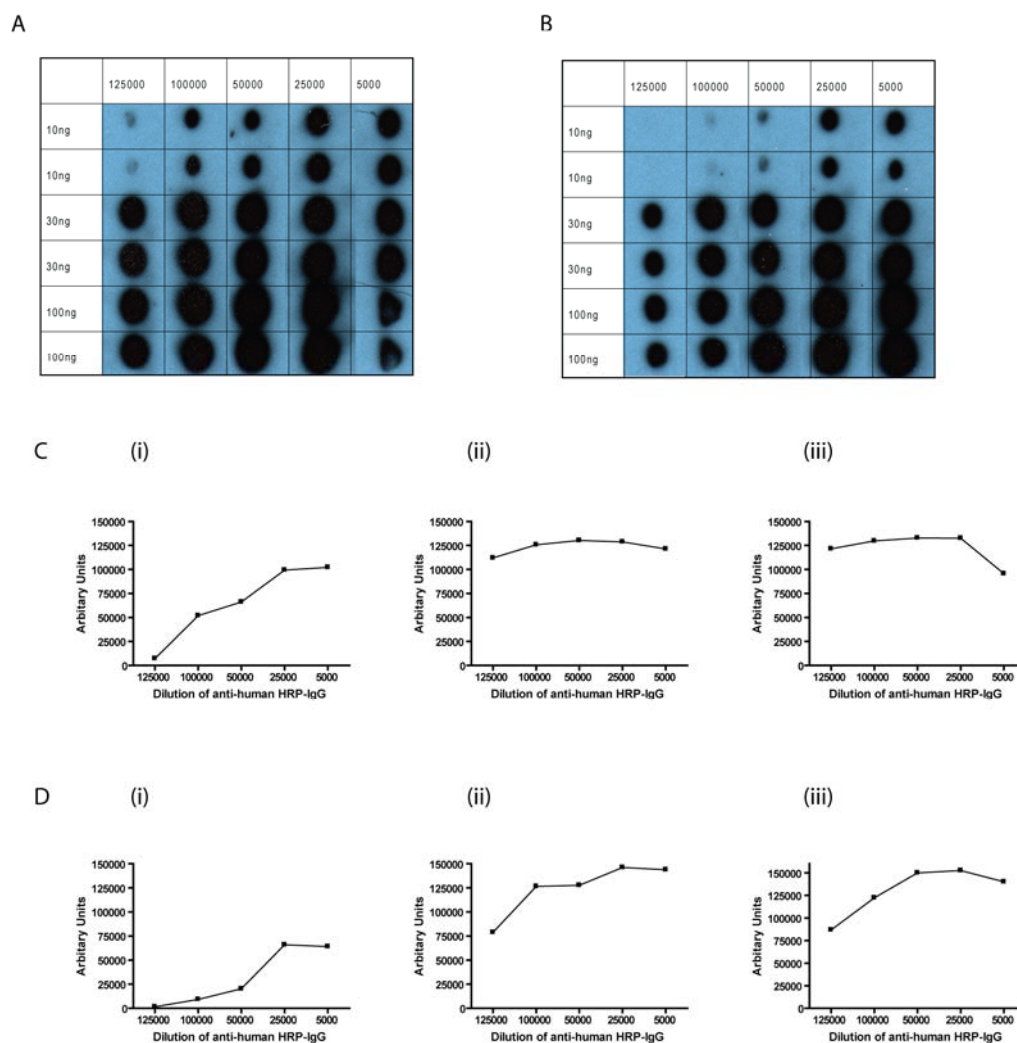
F. Serum 64841 assayed with initial preabsorption step

#### 2.2.2.4 Dilution of antibody

Assays were then carried out using decreasing concentrations of anti-Human HRP-IgG. Figure 2.5 illustrates data obtained from investigation of one serum, 070311, with known anti-GM1 IgG with titre on ELISA of > 12,500. Arbitrary units of intensity were measured using Image Quant Total Lab100 software and were averaged for each of the concentrations of lipids spotted; 10ng, 30ng and 100ng. The signal intensity appears saturated across all secondary antibody dilutions for spots obtained with 30ng and 100ng. However for 10ng lipid spots a curve results with anti-GM1 reactivity lost at lower concentrations of anti-Human HRP-IgG (see Figure 2.5. (C)(i) & (D)(i)). This was observed for serum diluted at 0.01% and 0.001% respectively.

10ng spot sizes were chosen as these were closest to the final lipid concentration used in ELISA. According to the ELISA protocol 200ng of lipid is placed into an individual well, the area of which is estimated to be  $28.26\text{mm}^3$  ( $\pi.r^2 = 3.14 \times 9$ ). This gives a final concentration of  $7.07\text{ng}/\text{mm}^3$ . In order to estimate the area of the “spot” of lipid sprayed 10ng of diluted methylene blue was sprayed onto A4 paper. The image was scanned and the dimensions were obtained using Image J with resultant dimensions of 0.31 width and 0.9 length giving an area of  $0.279\text{mm}^3$ . This gives a final lipid concentration of  $35\text{ng}/\text{mm}^3$ .

As a result of these experiments the serum concentration chosen was 0.01% (good level of binding observed across all antibody dilutions) and the anti Human HRP-IgG dilution of 1/25000 was chosen as this concentration was associated with near maximal binding to the lipid antigen.



**Figure 2.5 Dilution of secondary antibody**

All data here is obtained from one serum, 070311, with known anti-GM1 IgG with titre on ELISA at > 12,500. GM1 is the only lipid assayed in this experiment.

A. 070311 diluted to 0.01%.

B. 070311 diluted to 0.001%.

C. (i) Quantification of 10ng spots in 0.01% serum

(ii) Quantification of 30ng spots in 0.01% serum

(iii) Quantification of 100ng spots in 0.01% serum

D. (i) Quantification of 10ng spots in 0.001% serum

(ii) Quantification of 30ng spots in 0.001% serum

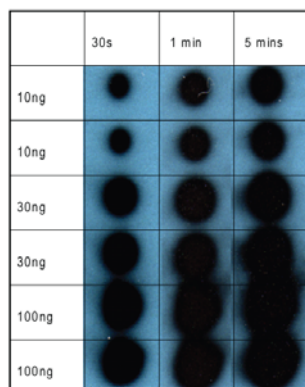
(iii) Quantification of 100ng spots in 0.001% serum

### 2.2.2.5 Quantitation of anti-lipid antibody

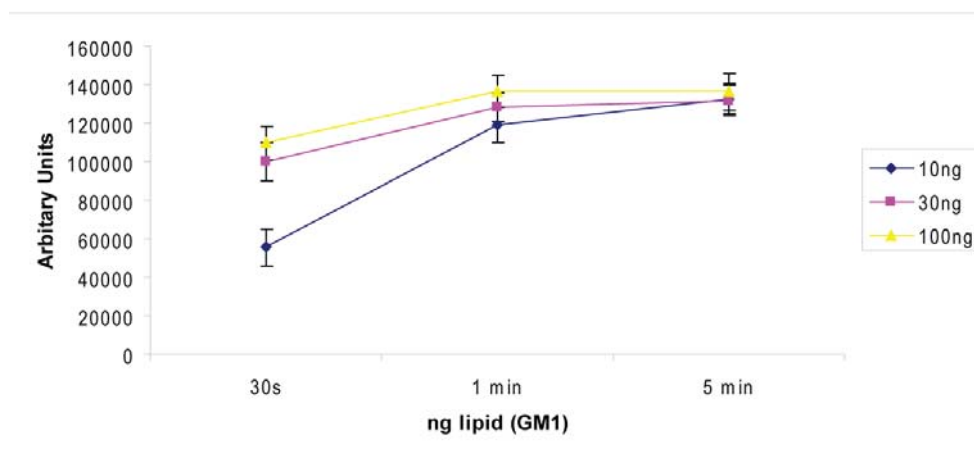
If autoradiographs are to be analysed by densitometry then it is essential to work in the range where the film gives a linear response to amount of radioactivity. This can be established by repeating blots at different amounts of exposures. Figure 2.6 illustrates data obtained using a serum with known high titre ( $> 12500$ ) against the lipid GM1. Here the serum has been probed on membranes with 10ng, 30ng and 100ng GM1 lipid spots. For the 10ng lipid spot there is a linear relationship between spot intensity (measured in arbitrary units) and duration of film exposure.

To ascertain the relationship for low titre anti-lipid antibodies a combinatorial array was used employing 45 potential target antigens per slide. Lipid combinations were premixed (w:w) and sonicated prior to printing. This template was designed by Dr.Simon Rinaldi. The serum 070536 was probed at 1 in 100 dilution with HRP IgG diluted at 1:25000 and successive exposures to film were obtained. The amount of lipid spotted in isolation and in complexes used was 10ng only. This serum is known to have high titre anti-GM1 and -GD1b IgG. Near maximum signal is observed at exposure time of 1 minute. For lower titre anti-lipid antibodies such as anti-GalC and anti-GalC/Chol complex antibodies a relationship between spot intensity and duration of film exposure is observed.

A



B



**Figure 2.6.** The effect of length of exposure of membrane to film on anti-lipid IgG intensity (measured in Arbitrary Units). All data here is obtained from one serum, 070311, with known anti-GM1 IgG with titre on ELISA at > 12,500.

A. Illustrative blots of serum 070311 diluted at 1:100 and assayed with anti-human HRP IgG 1:25000. 10ng, 30ng and 100ng of GM1 have been spotted in duplicate. The films were analyzed at exposures of 30 seconds, 1 minute and 5 minutes.

B. Quantification of anti-GM1 antibody for increasing amounts of lipid antigen and at exposures of 30 seconds, 1 minute and 5 minutes.



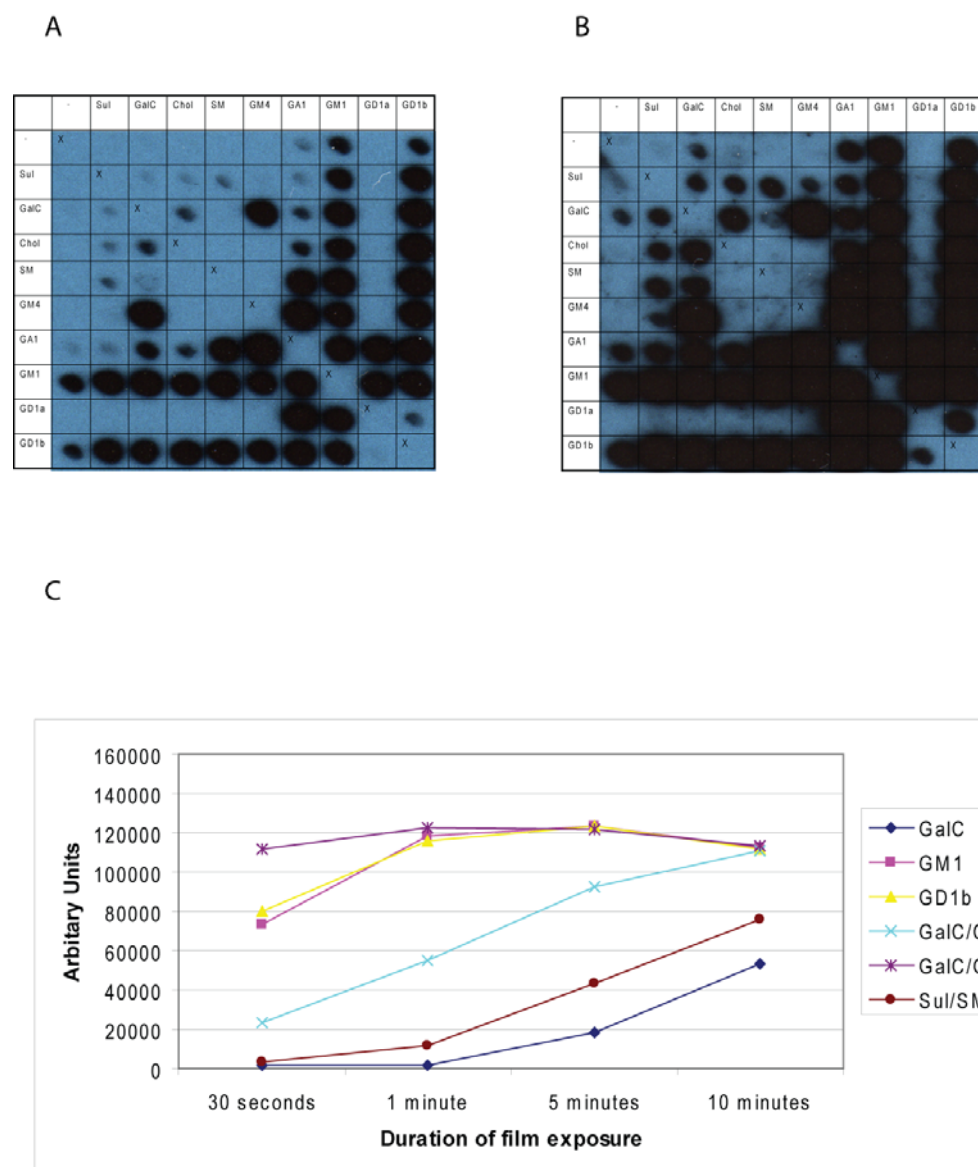


Figure 2.7. The effect of exposure of membrane to film.

A. Illustrative blot obtained after 30s film exposure to serum 070536. Row and column headings reveal the complex at each location.

B. Illustrative blot obtained after 5 minutes film exposure to serum 070536

C. Quantification of IgG to single lipid and complexes of lipids at increasing exposures to film

#### 2.2.2.5. Use of IgG as intrinsic positive control

Protein assays commonly use a universal protein such as actin to test as an inherent “positive control”. These inherent quality control measures can also be set as a maximum value against which all other blot intensities are expressed as a function/fraction. We tried to employ this in our array methodology using human derived IgG (Sigma, UK). Alongside columns of lipid antigens we sprayed equivalent concentrations of human IgG (Figure 2.8. (A) & (B)). We then probed with anti-human IgG-HRP as before. This then led to a splaying out of signal with a higher background signal. When the IgG was spotted (using a separate “contact” needle which makes direct contact with the membrane rather than spraying in a thin band) the resultant signal reduced in intensity very quickly (Figure 2.8. (C), (E)).

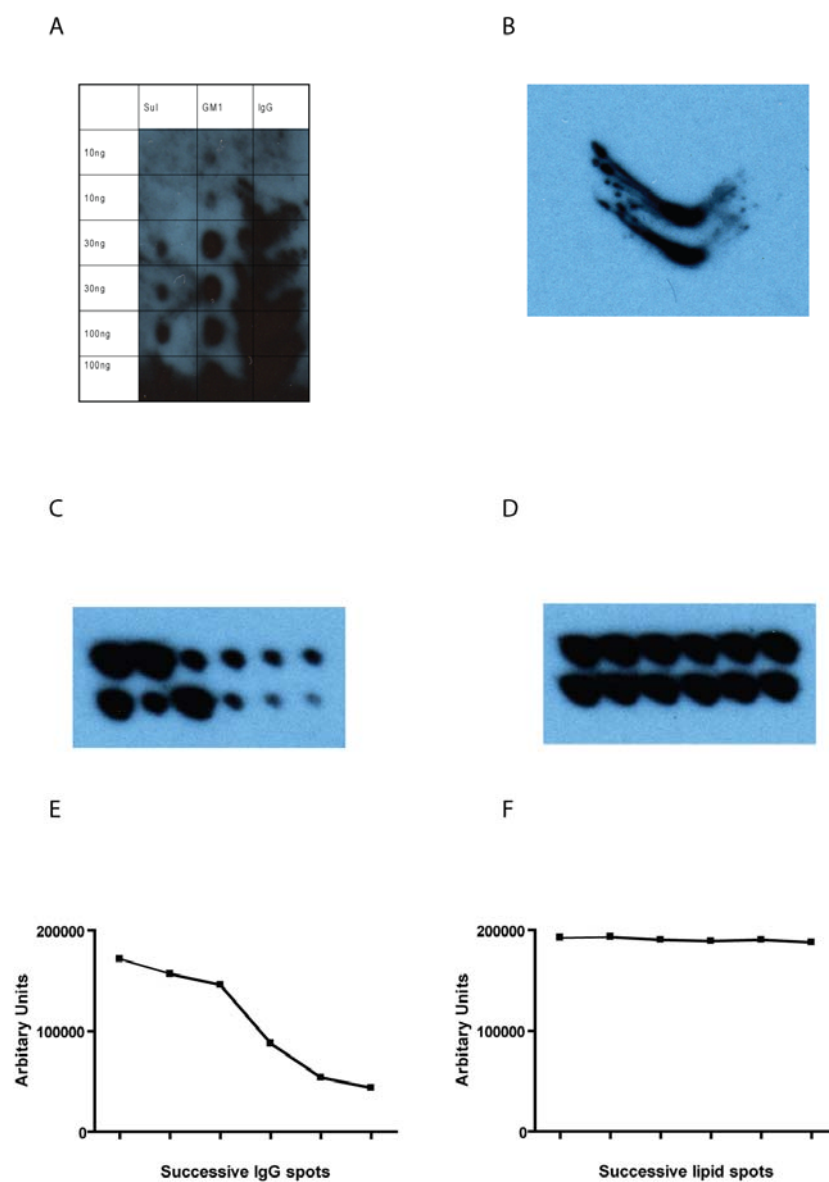


Figure 2.8. Protein spots

A. Illustrative blot obtained with human serum 070906 with known IgG to GM1 & sulfatide.

B. IgG sprayed onto membrane and probed with anti-Human HRP-IgG only

C. IgG spotted onto membrane and probed with anti-Human HRP-IgG only

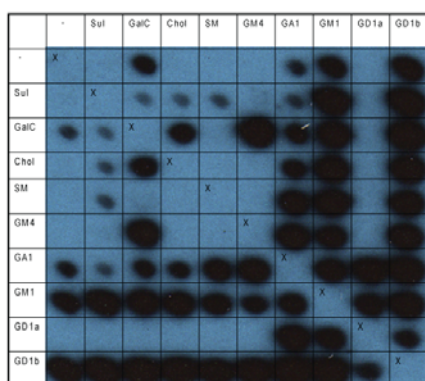
D. GM1 sprayed onto membrane and probed with anti-GM1 antibody and anti-Human HRP-IgG.

E & F. Quantification of protein and lipid spots (average of two vertical spots) in C & D respectively

#### 2.2.2.6 Investigation of membrane

We also ascertained that nitrocellulose membrane (Invitrogen, UK) did not serve as well as the PVDF membranes. Practically the nitrocellulose membranes were very friable and did not adhere well to the microscope slide. In addition the lipids appeared to adhere poorly to the nitrocellulose membrane. An example is shown in Figure 2.9.

A



B

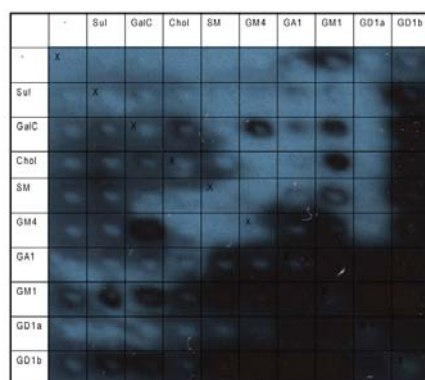


Figure 2.9. PVDF vs. nitrocellulose membrane

A. Illustrative combinatorial blot of serum 070500 on lipid array printed on PVDF membrane. As before row and column headings reveal the complex at each location.

B. Illustrative blot of serum 070500 on lipid array printed on nitrocellulose membrane

#### 2.2.2.7. Sequence of printing lipid

Separate experiments were carried out to ascertain if there was a difference in binding patterns between lipid pairs which were spotted separately onto the membrane (i.e. each single lipid was sprayed separately onto the PVDF membrane forming a complex only when the second lipid is sprayed, “overprinting”) and those which were premixed and sonicated in vials prior to printing.

It became apparent that whilst new anti-complex reactivity was still observed however the converse was not true. Those lipid partnerships which were inhibitory to IgG binding no longer had an attenuating effect on antibody binding. An illustrative example is depicted in Figure 2.10.

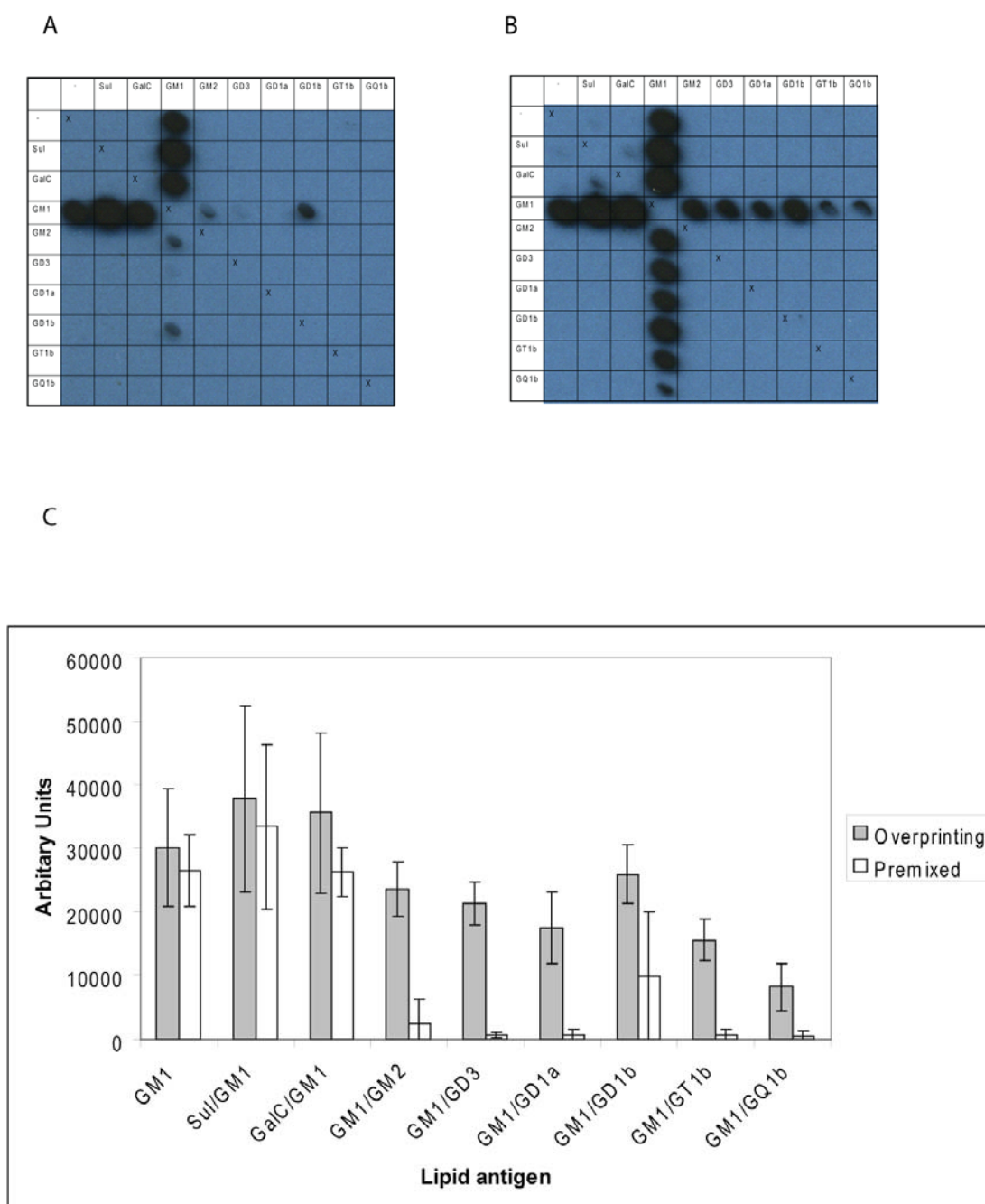


Figure 2.10. Comparison of printing methods: premixed Vs “overprinting”

A. Illustrative blot from GBS patient 223 using array created with premixed lipids

B. Illustrative blot from GBS patient 223 using array created using “overprinting” technique

C. Quantification of binding to GM1 and GM1 complexes using both techniques

#### 2.2.2.8. Storage of slides

To ascertain how stable the printed membranes were serum samples were assayed on membranes printed in the same “print run” on day 1. The assay (using the same serum, 070500) was repeated along 5 separate time points after the slides were printed; 1 hour, 1 day, 1 week, 2 weeks and 3 months. This particular serum (070500) had demonstrated high titre binding to GM1 (>12500) on ELISA. Between the time points of 1 and 2 weeks there was a considerable reduction in signal intensity observed for IgG directed against single and complex lipids (Figure 2.11). In order to exclude this as a variability factor all slides were thereafter assayed 1 hour after the membranes were printed with lipid.



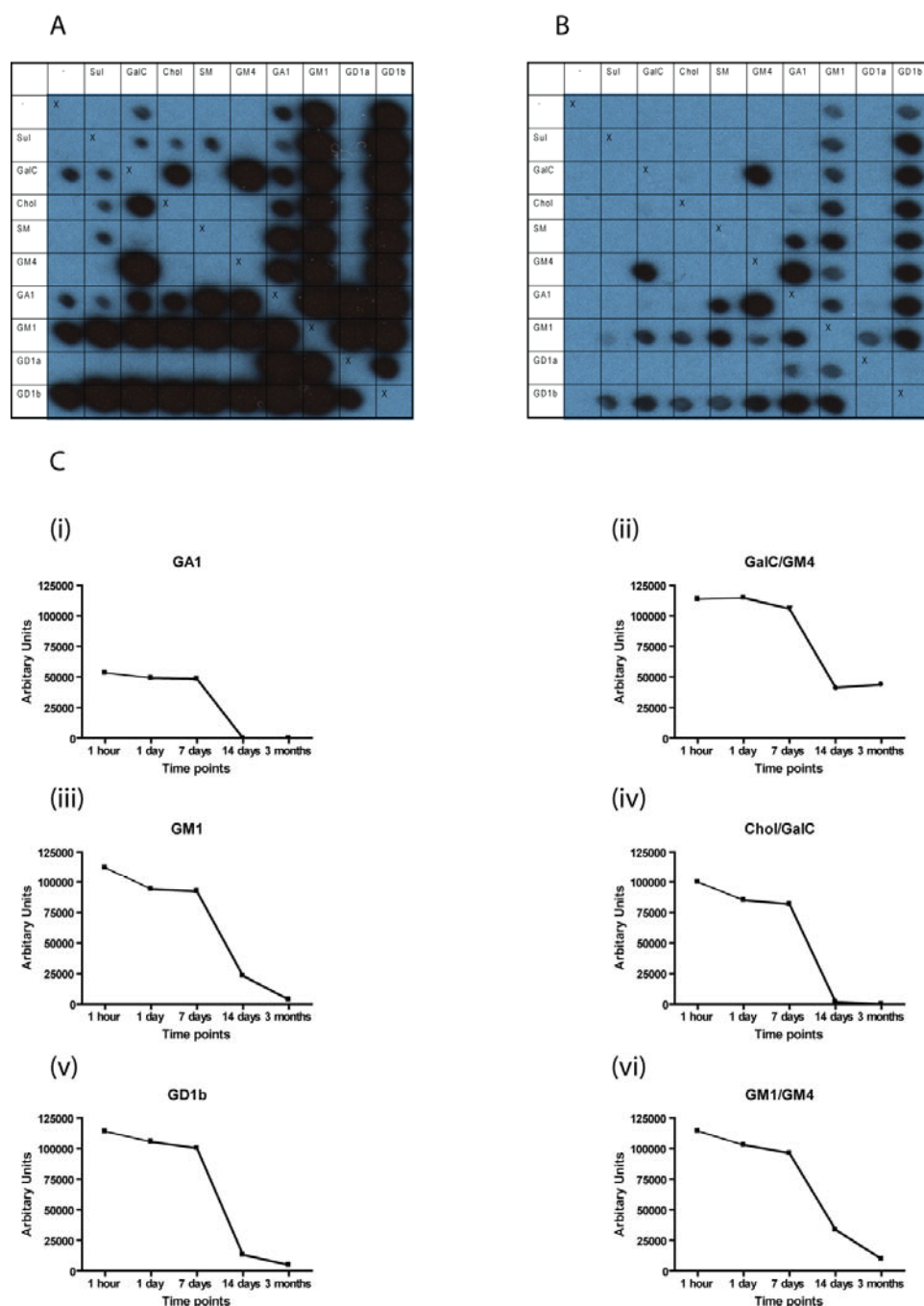


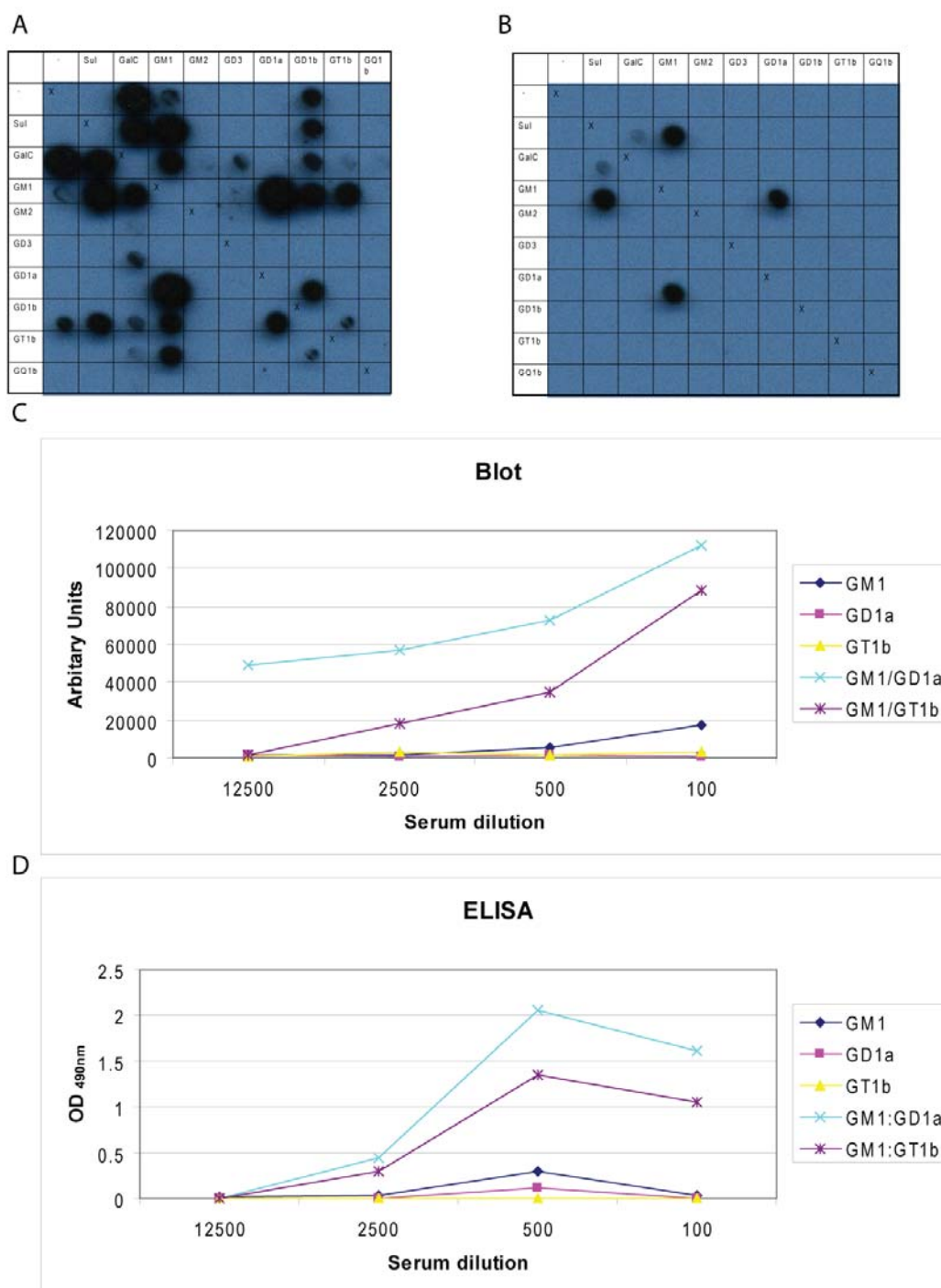
Figure 2.11. Effect of storage of printed lipid slides

A & B illustrative blots obtained with serum 070500 when slides used after 1 hour and after a period of 3 months respectively.

C (i)-(vi) Quantification of binding to single lipids and complexes of lipids across 5 different time points.

### 2.2.3 Detection of anti glycolipid complex antibodies

In parallel with these preliminary studies on the experimental protocol we investigated if the method would allow the detection of antibodies directed against a pair or complex of lipid antigens. Arrays of 10 lipids and all their 1:1 combinations were spotted in duplicate on the PVDF membranes (Figure 2.12). The lipids probed included sulfatide (Sulf), galactocerebroside (GalC), GM1, GM2, GD3, GD1a, GD1b, GT1b and GQ1b. Membranes were then probed, as before, using anti-Human HRP-IgG at a concentration of 1/25000. Our array method appeared to be as sensitive as the ELISA method in detecting complex antibodies. Further studies involving larger patient cohorts would be undertaken to investigate this further.



**Figure 2.12. Anti complex antibodies detected on ELISA and array**

(A) & (B) illustrative blots obtained from serum of GBS patient at 1/100 and 1/12500 dilutions respectively. Row and column headings reveal the complex at each location. “Xs” represent the negative controls (methanol) which act as a line of symmetry for duplicate spots within the same membrane.

C. Quantification of binding using array method to single lipids and lipid complexes

D. Quantification of binding using ELISA to single lipids and lipid complexes

#### 2.2.4 Inter and intra-assay variation

As a result of protocol work to date the experimental protocol was altered as follows:

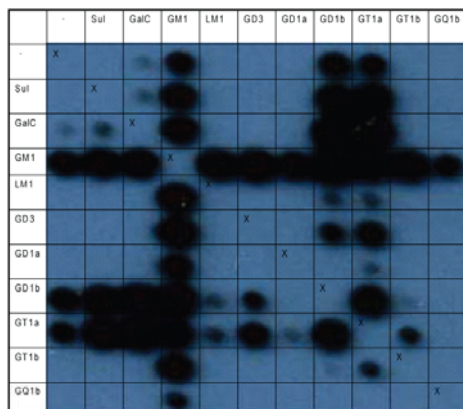
Membranes were cut into equal sizes with a scalpel (2.8cm by 2.5 cm) and adhered to glass microscope slides (VWR International, UK). Scotch tape proved to be poorly adherent to the glass slides therefore we used UHU glue (Ryman, UK) as an adhesive agent. A hydrophobic marker was then used to outline the membrane. The membranes were blocked for 1 hour in 2% FAF BSA/PBS (Europa Bioproducts Ltd, UK). Slides were immersed in glass Copeland jars for blocking and washing steps. BSA was then discarded and slides were briefly immersed in PBS. Thereafter 500ul of diluted serum (1:100 in 0.1% FAF BSA/PBS) was then added per slide. Each slide was incubated for 1 hour at 4°C. Slides were then washed. This entailed incubating the slides at room temperature in PBS on a shaker (75rpm) for 30 minutes. The solution was refreshed every 5 minutes. Secondary antibody (polyclonal rabbit anti-human IgG, Dakocytomation, Denmark) was then diluted to 1:25000 in 0.1% FAF BSA/PBS and slides were incubated for 1 hour at 4°C. After this incubation a further series of washes was undertaken. After a final wash in distilled H<sub>2</sub>O, ECL solution (Amersham ECL Plus, GE Healthcare, UK) was applied to each slide for 5 minutes. Slides were then placed in a cassette with trimmed transparency paper overlying. Film was then placed over this transparency paper in the dark room and exposures were taken at 1 minute. Films were digitalized using a flatbed scanner (Epson DX 6000). TIFFs were then quantified using appropriate analytical software (Image Quant Total Lab100, UK) which measures the pixel intensity of individual spots. The methanol spots on each individual slide were selected as negative controls. The Total Lab software applies this average as the background intensity.

Inter-assay variability was then determined by assaying 5 different membranes with the human serum, 070536, on different occasions on a lipid template of sulfatide (sulf), galactocerebroside (GalC), GM1, LM1, GD3, GD1a, GD1b, GT1a, GT1b and GQ1b. This serum was known to have anti-GM1 and -GD1b antibody on ELISA with titres > 1/12500. Two example processed blots are illustrated in Figure 2.13 (A). Using this method the coefficients of variation (CVs) for antibody directed against single lipids were measured at 9.92% (GM1), 6.67% GD1b and 6.65% (GT1a). The observed CVs were similar for antibody directed against complexes of lipids e.g. 7.73% GT1a/GD1b.

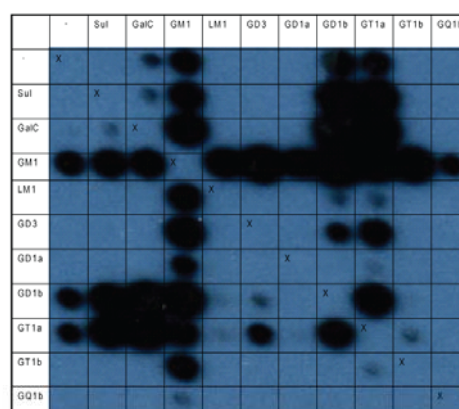
To determine the intra-assay coefficient of variance 4 separately prepared membranes were incubated with the human serum, 070500 (Figure 2.13 (B)). The lipid template employed included sulfatide (sulf), galactocerebroside (GalC), cholesterol, sphingomyelin, GM4, GA1, GM1, GD1a and GD1b. This serum was known to have anti-GM1 and -GD1b antibody on ELISA with titres > 1/12500. The coefficients of variation were measured at 8.91% and 7.74% for GM1 and GD1b respectively.

A

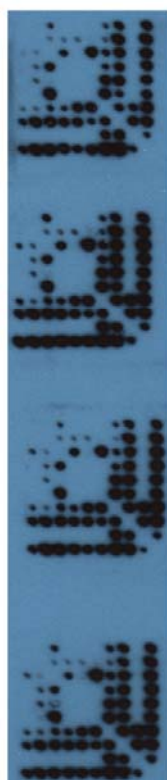
(i)



(ii)



B (i)



(ii)

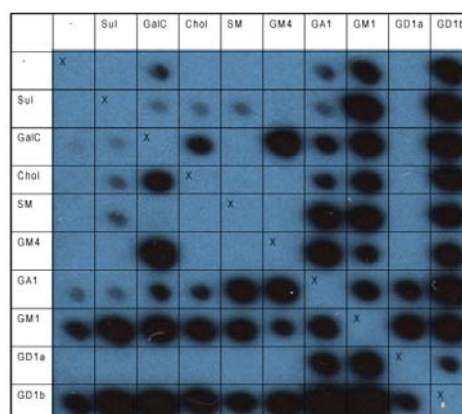


Figure 2.13. Inter- and intra-assay variation

A. Inter-assay variation; 2 examples of illustrative blots of serum 070536

B. Intra-assay variation; illustrative blots of serum 070500

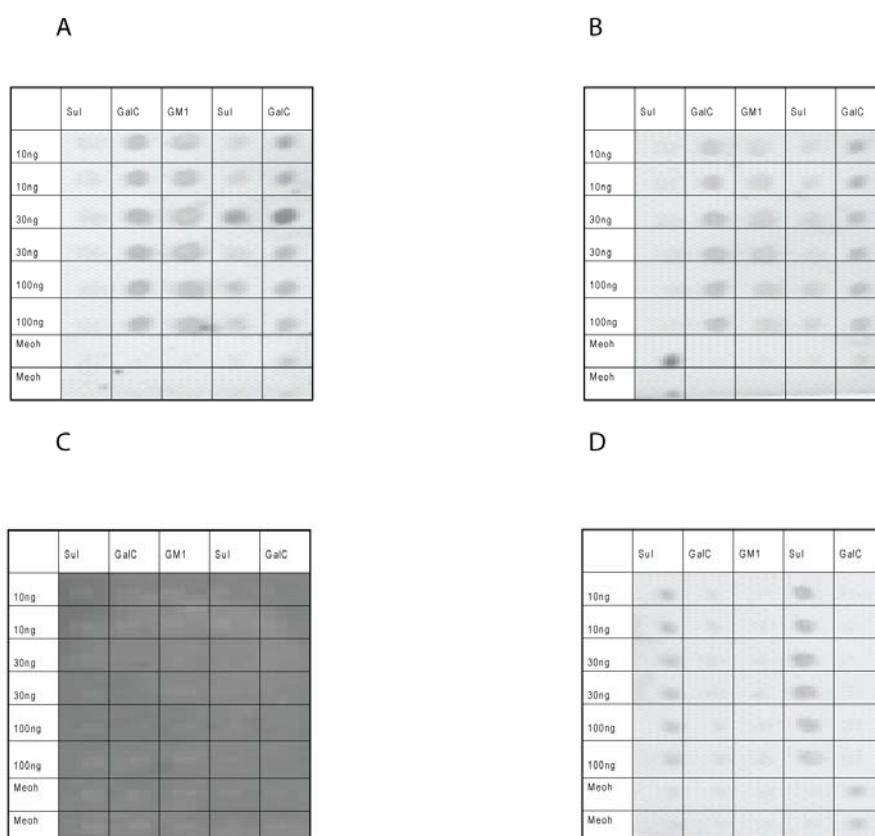
### 2.2.5. Development of fluorescent protocol

A fluorescent based technique could have advantages such increasing the number of slides “assayable” in one experiment and also potentially broadening the range of intensity. A chemifluorescent protocol is analogous to chemiluminescence in utilizing an enzyme to cleave a substrate but generates a fluorescent product rather than light in the process. Initially we investigated this technique using the anti-GM1 mouse monoclonal antibody, DG2 with various fluorescent anti mouse IgG antibodies (Figure 2.14).

Sulfatide, galactocerebroside and GM1 were printed onto PVDF covered slides as before. Slides were blocked and probed with primary antibody as before (10ug/ml in case of mouse monoclonal) and several fluorescent anti IgG were investigated. The slides were scanned using a Typhoon 9400 scanner (GE Healthcare, UK) with spectra set at 633 for absorption and 670 for emission. DG2 appeared to bind to all lipids using Cy5 (origin) and TRITC (origin) secondary IgG. This is not unexpected given what we know about the binding capabilities of this monoclonal antibody (Townson et al. 2007). Unsurprisingly FITC (which absorbs at a spectra of 494 and emits at a spectra of 521) did not produce any useful images.

We then next investigated human serum 074154 with known high anti sulfatide titre on ELISA (>12500) using AF 647 labelled Goat anti Human IgG (Invitrogen). AF 647's spectra virtually matches that of CY5 with an absorptive spectra of 650 and emission spectra of 668. This serum appears to bind with some specificity to the sulfatide however it was noted that there was some non specific binding to the methanol only spots (Figure 2.14 (D)).

We next investigated the role of several different blocking agents in reducing this binding to the methanol only spots. Even when the membranes were not incubated with both primary and secondary antibody there was reactivity seen for individual lipid spots indicating an inherent fluorescent quality to the lipids (Figure 2.15).



**Figure 2.14 Initial development of fluorescent based technique**  
 Images were obtained using Typhoon scanner (Absorption 633, Emission 670, Voltage 600 and resolution of 200)

A. Incubation with DG2 antibody and CY5 (1/300) labelled anti mouse IgG  
 B. Incubation with DG2 antibody and TRITC labelled anti mouse IgG  
 C. Incubation with DG2 antibody and FITC labelled anti mouse IgG  
 D. Incubation with serum 074154 (with known IgG to sulfatide on ELISA) and AF 647 labelled anti Human IgG



A

	Sul	GalC	GM1
10ng			
10ng			
30ng			
30ng			
100ng			
100ng			
Meoh			
Meoh			

B

	Sul	GalC	GM1
10ng			
10ng			
30ng			
30ng			
100ng			
100ng			
Meoh			
Meoh			

C

	Sul	GalC	GM1
10ng			
10ng			
30ng			
30ng			
100ng			
100ng			
Meoh			
Meoh			

D

	Sul	GalC	GM1
10ng			
10ng			
30ng			
30ng			
100ng			
100ng			
Meoh			
Meoh			

Figure 2.15. Incubation of slides with blocking agents only

A. FAF BSA

B. Regular BSA (Sigma, UK)

C. 2% non-fat dried milk

D. Superblock buffer

I next tried a streptavidin based technique in an effort to try and reduce the level of fluorescence. Streptavidin is a 52,800 Dalton tetrameric protein buffered from the bacterium *streptomyces avidinii*. It has a very high affinity for biotin. I preincubated biotin with the primary antibody (diluted human serum 1/100). Initial results were very promising with no background or methanol binding however after successive experimental runs binding to the methanol only spots was observed (Figure 2.16).

I then incubated slides with blocking agents only (again missing out the incubation with primary antibody and secondary antibody) and demonstrated that there was an inherent fluorescent quality to the lipids (Figure 2.17). Because of this the fluorescent protocol was abandoned.

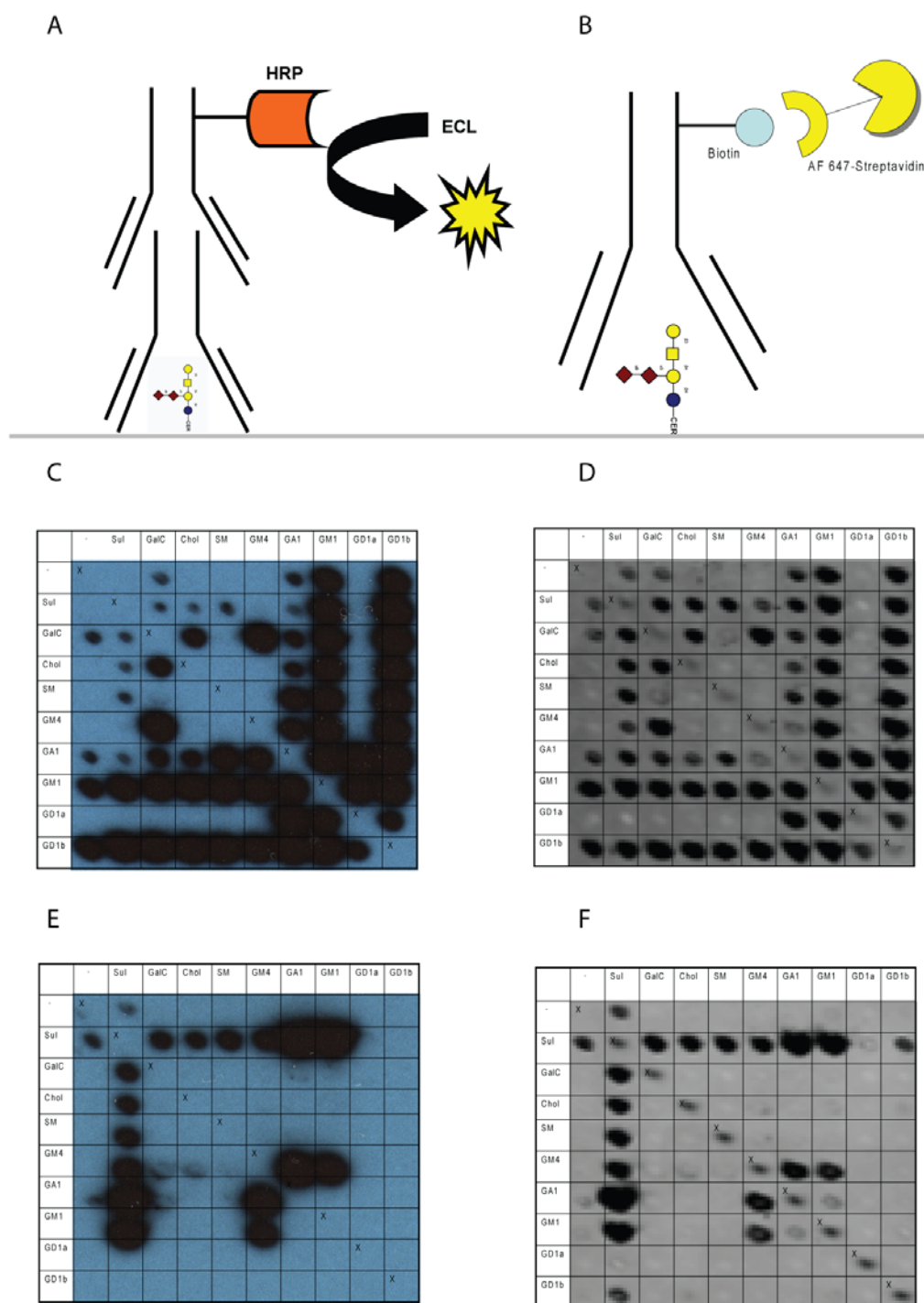


Figure 2.16. Biotin-streptavidin protocol

A. Experimental protocol for blots C &amp; E

B. Experimental protocol for blots D &amp; F

C. Serum 070500

D. Serum 070500

E. Serum 072289

F. Serum 072289

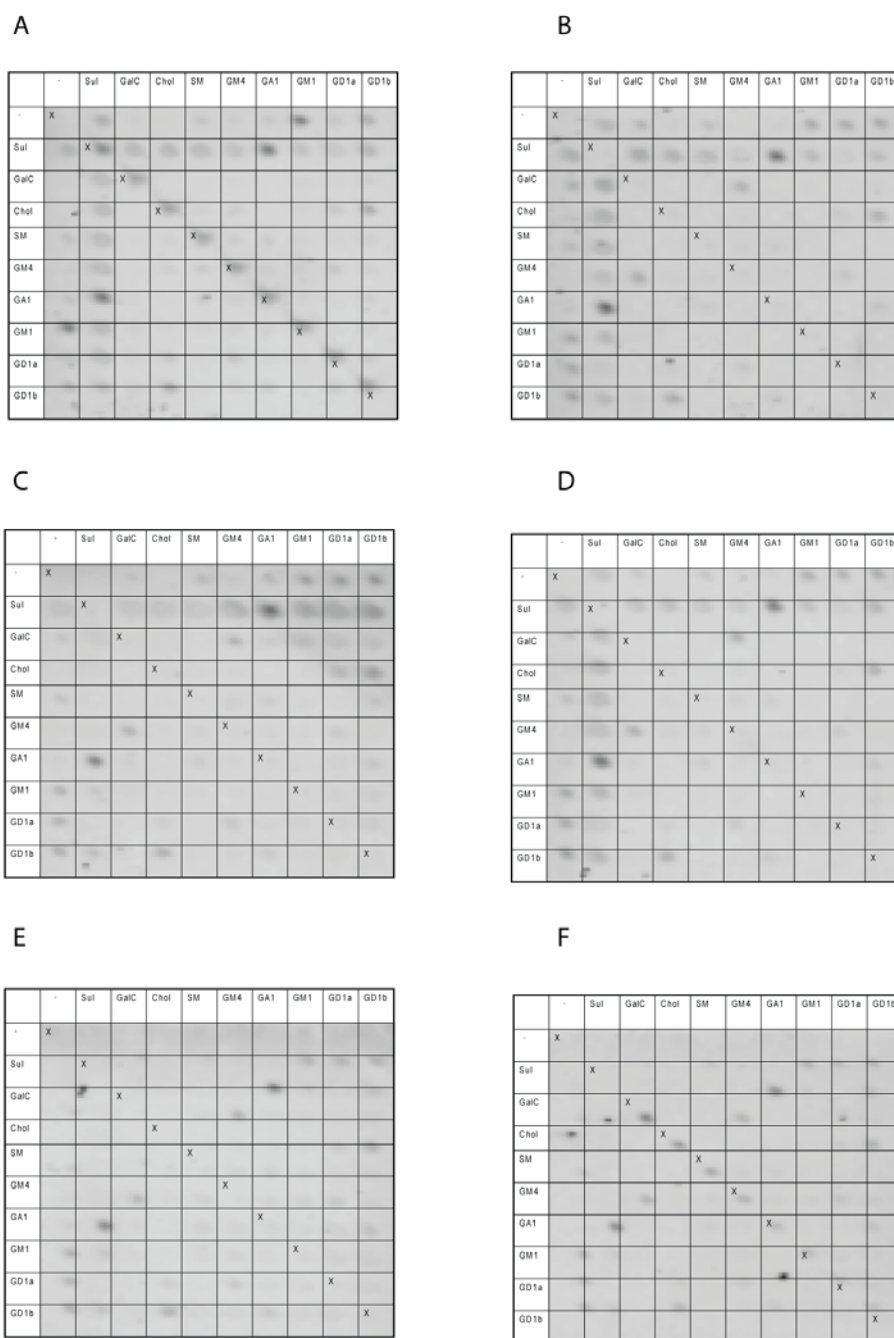


Figure 2.17. Demonstration of fluorescent nature of PVDF membrane

A. Slide scanned after incubation with FAF BSA

B. Slide scanned after incubation with FAF BSA- new source of methanol used when printing this slide

C. Slide scanned after incubation with Biotin only

D. Slide scanned after incubation with Streptavidin only

E. Slide scanned after incubation with Casein

F. Slide scanned after incubation with Superblock

### 2.3. Discussion

Due to the inherent fluorescent nature of the PVDF membranes I abandoned the fluorescent protocol and decided to use the HRP based technique for our series of investigations. I used a protocol using serum diluted at 1:100 with anti-human HRP IgG diluted at 1:25000. Successive film exposures were obtained but data presented in subsequent chapters is primarily from film exposures taken at 1 minute. I chose this film exposure time as it is easily reproducible and associated with near maximum signal of high-titre (by ELISA) anti-ganglioside antibody. Longer length of film exposures were associated with much higher background readings and “bleeding out” of signal from high intensity spots to adjacent negative spots.

The next aim was to investigate the profile of anti-glycolipid complex antibodies in several different patient populations including GBS, CIDP and MS. In addition I also wanted to compare the new method to the long established ELISA technique in the ability to detect anti-lipid antibody.

## Chapter 3. Guillain-Barré syndrome (GBS)

### 3.1. Introduction

As outlined in Chapter 1 gangliosides are highly enriched in cell surface membranes in both the central and peripheral nervous systems and have long been considered to be the likely antigenic target in the acute polyradiculoneuropathy Guillain-Barré syndrome (GBS). The observation that *cis* interactions of neighbouring gangliosides could influence the binding capabilities of anti-glycolipid antibodies was reported initially by Kaida et al using ELISA and TLC techniques (Kaida et al. 2004). I have employed the combinatorial lipid array technique to investigate further the anti-glycolipid and anti-glycolipid complex antibody specificities in sera from a large cohort of GBS patients. I collaborated with colleagues in Rotterdam who had collected a cohort of GBS patients ( $n=180$ ) and a smaller cohort of healthy controls ( $n=20$ ). Our Dutch colleagues had relevant clinical and paraclinical information (e.g. neurophysiological data such as GBS subtype; AIDP, AMAN etc) was available in a SPSS database.

### 3.2. Methods

#### 3.2.1. ELISA

Our Dutch colleagues had investigated the anti-ganglioside antibody (AGA) specificity (via ELISA) of a large cohort of GBS patients. They investigated IgG serum responses to the gangliosides GM1, GD1a and GQ1b. Titres were available for GM1 AGA but were not for GD1a or GQ1b. ODs were unavailable for all ganglioside AGAs. For ELISA experiments carried out in Glasgow the specificities of sera for gangliosides were assayed by the ganglioside ELISA as described in section 2.2.3. A positive binding result was considered to give an OD<sub>490nm</sub> of 0.1 or above.

### 3.2.2. Combinatorial array

Using the Camag Automatic TLC Sampler 4, I assembled an array of individual glycolipids and their 1:1 combinations (complexes) spotted onto polyvinyl-difluoride (PVDF) membranes affixed to microscope slides. I investigated the serum IgG response to the lipids sulfatide (Sulf), galactocerebroside (GalC), GM1, LM1, GD3, GD1a, GD1b, GT1a, GT1b, GQ1b and all their possible 1:1 complexes thus comprising 55 target antigens in total. Complexes were premixed and sonicated for 3 minutes prior to printing. Details of where lipids were purchased are outlined in Chapter 2, Table 2.1. I probed these prepared slides with 180 GBS serum samples and 20 serum samples from healthy controls. Each serum was assayed at a concentration of 1:100 unless otherwise stated. Antibody binding to specific lipids was then detected using standard chemiluminescence and autoradiography using the protocol as outlined in Chapter 2; 2.2.4. Films were digitalized using a flatbed scanner (Epson DX 6000). TIFFs were then quantified using appropriate analytical software (Image Quant Total Lab100, UK) which measures the pixel intensity of individual spots. The methanol spots on each individual slide were selected as negative controls. The Total Lab software applies this average as the background intensity.

### 3.2.3. Thin Layer Chromatography (TLC)

Aluminium TLC plates were sliced in half (smooth edges retained) using a scalpel. 25ug of individual gangliosides (1mg/ml) were spotted using the Camag Automatic TLC Sampler 4 onto high performance TLC plates (origin). The samples were then allowed to dry for 1 hour. TLC running buffer was then prepared using glass measuring cylinders using chloroform, methanol

and 0.2% calcium chloride (50:45:10; v/v/v). 2 pieces of blotting paper were cut; one piece measuring half the height of the tank and the other measuring the full height of the tank. These were placed at separate ends of the tank. After placing the lid securely on the tank this was then left to equilibrate for 5 minutes. The TLC plate was then placed in the tank between the two pieces of blotting paper and allowed to run. After 30 minutes the TLC plate was then removed and allowed to dry before cutting into strips. At this stage strips were either stained with resorcinol stain or probed with serum. Resorcinol stain was prepared and sprayed lightly onto the individual strips in the fume hood. This stain was prepared using 2.5mls of 2% resorcinol, 62.5ml of  $\text{CuSO}_4$ , 2.437ml  $\text{dH}_2\text{O}$  and 20ml concentrated HCL (added last). Gangliosides were then visualized by the application of heat to individual strips.

For staining with serum or monoclonal antibody strips were not sprayed with resorcinol. Firstly they were coated with PIBM (polyisobutylmethacrylate) (Sigma Aldrich, UK) in n-hexane as a fixative for 1 minute. PIBM is dissolved in chloroform to 2.5% before further dilution to 0.4% in n-hexane. After air drying for 30 minutes the strips were then blocked for 1 hour in 2% FAF BSA/PBS (origin). BSA was then discarded and strips were briefly immersed in PBS. Thereafter 5ml of diluted serum (1:1000 in 0.1% FAF BSA/PBS) or monoclonal antibody (10ug/ml in 0.1% FAF BSA/PBS) was then added per strip. Each strip was incubated for 1 hour at 4°C. Strips were then washed. This entailed incubating the strips at room temperature in PBS on a shaker (75rpm) for 30 minutes. The solution was refreshed every 5 minutes. Secondary antibody (HRP conjugated goat anti-mouse IgG used in monoclonal experiments and HRP conjugated anti-human IgG used in human sera experiments) (Dakocytomation) was then diluted to 1:3000 in 0.1% FAF BSA/PBS and strips were incubated for 1 hour at 4°C. After this incubation a further series of washes was undertaken. After a final wash in distilled  $\text{H}_2\text{O}$ , ECL solution (Amersham ECL Plus, GE Healthcare, UK) was applied to each strip for 5 minutes. Strips were then placed in a cassette with trimmed transparency paper overlying. Film was then placed over this transparency



paper in the dark room and exposures were taken at 1 minute. Films were digitalized using a flatbed scanner (Epson DX 6000).

### 3.2.4. Patients

Patient characteristics of this cohort are outlined in Table 3.1. Certain clinical (e.g. distribution and severity of weakness) and paraclinical details (EMG classification, Table 3.2.) were available for this cohort in a SPSS database provided by our Dutch colleagues (van Koningsveld et al. 2004). No demographic information was available for the sera obtained from healthy controls.

Table 3.1. Characteristics of GBS patients

	GBS cohort
<i>N</i>	180
Sex	
Male	103
Female	77
Age (y)	
Median	57
Range	40-68

Age and sex for the patient subgroups. Data are presented as median (IQR-interquartile range).

Table 3.2. EMG classification of GBS cohort

EMG Classification	Frequency
AIDP	56
AMAN	4
Equivocal	85
Unexcitable	4
Unavailable	31

### 3.2.5 Statistical analysis

ANOVA analyses were used to compare anti-glycolipid levels between more than three groups. The Mann-Whitney test was used to compare anti-glycolipid antibody levels between two groups. Paired t tests were used to detect if anti-complex antibodies were of higher intensity than antibodies to the contributing single lipid. Differences in proportions were tested for significance by chi-squared test and Fisher's exact test where appropriate. These analyses were performed with PRISM4 (GraphPad software, USA). Raw data was logarithmically transformed (log 2) and heatmaps, hierarchical clustering and further ANOVA analyses were performed by Dr Gabriela Kalna of the University of Glasgow using Partek Geometrics Suite (Partek Incorporated, USA).

## 3.3. Results

### 3.3.1 ELISA

39/180 (21.66%) of the GBS cohort were observed by our Dutch colleagues to have AGA (illustrated in Figure 3.1 (A)). Most patients demonstrated a monospecific response to one lipid only ( $n=29$ ). 10 patients displayed polyreactivity to gangliosides with 5 sera displaying IgG reactivity to GM1 & GD1a, 4 sera displaying IgG reactivity to GD1a & GQ1b and 1 serum displaying IgG reactivity to GM1 & GQ1b (Figure 3.1. (B)).

### 3.3.2. ELISA vs. combinatorial array

I then compared our combinatorial array method to ELISA. Inter- and intra-assay variation did not differ between the two methods (see chapter 2, section 2.2.4). For each ELISA and combinatorial blot experiment performed in Glasgow, a serum with known IgG reactivities to the gangliosides GM1,

GD1b and GT1a was used as a positive control in each batch of experiments performed.

Our Dutch colleagues reported that 24/180 patients from the GBS cohort were positive for IgG GM1 antibody. The correlation between the GM1 ELISA titres and the average spot intensity for GM1 obtained with the blot method, whilst positive, was modest (Figure 3.2. (A),  $r_s = 0.4304$ ,  $p < 0.0001$ ).

Figure 3.2. displays correlation and Bland-Altman graphs for ELISA and array data observed. Correlation quantifies how well X and Y variables vary together. As the data is not normally distributed Spearman correlation was employed. Two-tail p values were all significant demonstrating a positive correlation between the two methods.

However  $r_s$  measures the strength of a relation between two variables, not the agreement between them. Bland-Altman plots are a more informative way at comparing two assay methods (Bland et al. 1986). The graph is constructed so the difference between the two measurements (Y axis) is plotted against the average of the two measurements (X). As our two methods have different units of measurement, optical density and arbitrary units of intensity, the raw data was normalised. The highest value observed in each respective dataset was set as 100% with all other values expressed as a percentage. Whilst this is not ideal this method does give us a better feel of the agreement between the two methods. The observed spread of data on either side of the mean difference would appear to agree with the modest correlation between the two methods. (see Figure 3.2. (B)).

A proposed mechanism for the disparity in the ELISA and blot findings could be the age of these serum samples. Some of these samples were over 10 years old and sample degradation could lead to a reduction in AGA titre. In addition each sample had been assayed with ELISA when patients presented separately. This means that ELISA experiments were assayed potentially with different batches of lipid antigen and experimental reagents introducing additional variability. Therefore I then repeated the IgG GM1

ELISA in all 180 GBS & 20 healthy control samples in Glasgow. The correlation between the Glasgow ELISA with the blot method was similar (Figure 3.2. (B),  $r_s = 0.4428$ ,  $p < 0.0001$ ) to the correlation between the Dutch performed ELISA and the combinatorial blot method.

Although it is noteworthy that the titres obtained in Glasgow for IgG directed against GM1 were lower than those obtained in Holland. When specifically comparing the titres for only those patients positive on ELISA, there was a statistically significant reduction in titres between the two assays (Figure 3.3., 2 tailed paired t test,  $p = 0.0025$ ). Despite this when compared directly, the correlation between the GM1 ELISA performed in Holland and Glasgow was strong with an  $r_s$  of 0.9158 suggesting that there is a true difference between findings on ELISA and the combinatorial blot for individual serum samples. However all of the samples detected on the Dutch ELISA as demonstrating reactivity to GM1, did display reactivity on the blot to a variety of both single and pairs of lipid antigens (see Table 3.4).

As the same lipid stocks were employed as antigens in the Glasgow ELISA and the combinatorial blot, these two methods were directly compared. When using the ELISA method a positive binding result was considered to give an  $OD_{490nm}$  of 0.1 or above. Similarly with the combinatorial method a positive binding result was considered to give an intensity of 10,000 arbitrary units (measured with Total Lab software) or more. All intensities of 10,000 were double checked by eye and were associated with a positive blot on the radiograph. There were no intensities above 10,000 attributed to background activity only.

20/180 GBS samples demonstrated IgG against GM1 with  $ODs > 0.1$  when assayed via ELISA in Glasgow. None of the healthy control samples demonstrated binding to GM1. Interestingly only 13 of these GM1 ELISA positive samples demonstrated binding to GM1 using the combinatorial blot method explaining the modest correlation between the two methods (Figure

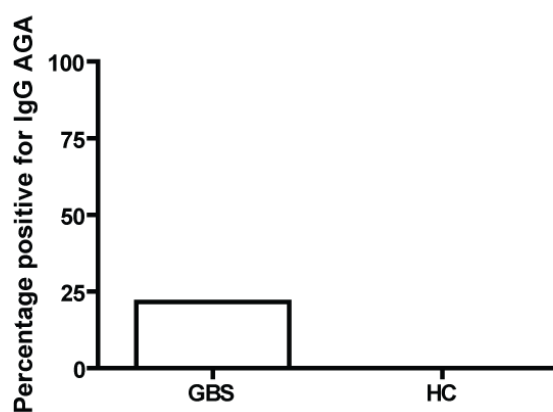
3.2. (B),  $r_s = 0.4428$ ,  $p < 0.0001$ ). Example blots are illustrated in Figure 3.4. Two patterns of antibody binding were observed. The first pattern is illustrated in Figure 3.4. (A) & (B) where antibody binding occurs against GM1 when presented singly and when complexed with most other lipid antigens. The second pattern is illustrated in Figure 3.4. (C) & (D) where anti-GM1 binding is inhibited when GM1 is complexed with specific lipids including LM1, GD1a, GT1b and GQ1b.

The 9 remaining samples, which were positive on ELISA for GM1 and negative for GM1 on the blot were not however completely negative on the combinatorial blot. All 9 samples demonstrated binding to a variety of single lipids and complexes of lipids (Table 3.4.).

Five of these samples demonstrated IgG reactivity to other single glycolipids (Figure 3.5 (A)-(E)), with four demonstrating reactivity to glycolipids only when complexed with sulfatide (Figure 3.6). Interestingly 6/9 of the samples which did not recognize GM1 on ELISA, bound to the complex created by sulfatide and GM1 (Figure 3.5 (C) & (E) and Figure 3.6. (A)- (C)). 3 of these 6 samples bound other single glycolipids including LM1, GD1a & GT1a and sulfatide. However two sera only bound to complexes with no demonstrable binding to single lipids (Figure 3.6. (A) & (B)).

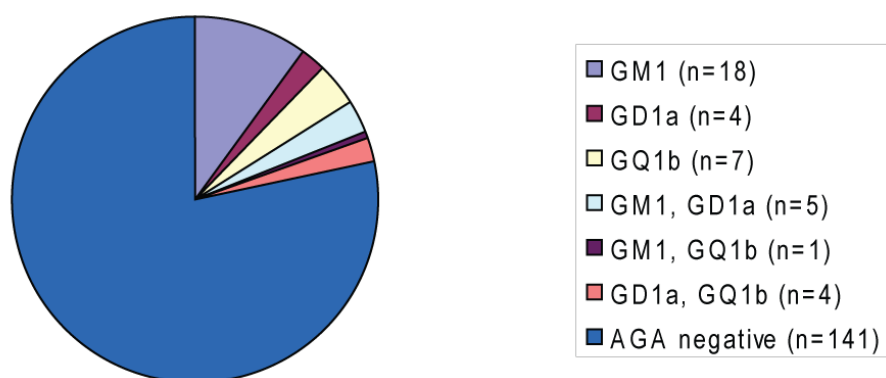
The converse observation was also described in two sera, where binding to GM1 was demonstrable on the combinatorial blot but negative on ELISA performed in Glasgow (Table 3.4. and Figure 3.7. (A)&(B)). Although when both of these sera were initially investigated via ELISA in Holland both demonstrated titres of IgG GM1 antibody of 400 and 1600 respectively.

A



B

**Anti ganglioside antibody profile in GBS cohort  
(*n*=180)**



**Figure 3.1. Anti-ganglioside antibody (AGA) findings in the GBS cohort by colleagues in Holland**

A. Frequencies of AGAs in Dutch cohort. AGAs were observed in 39/180 patients (21.66%).

B. Pie chart documenting breakdown of these AGA specificities

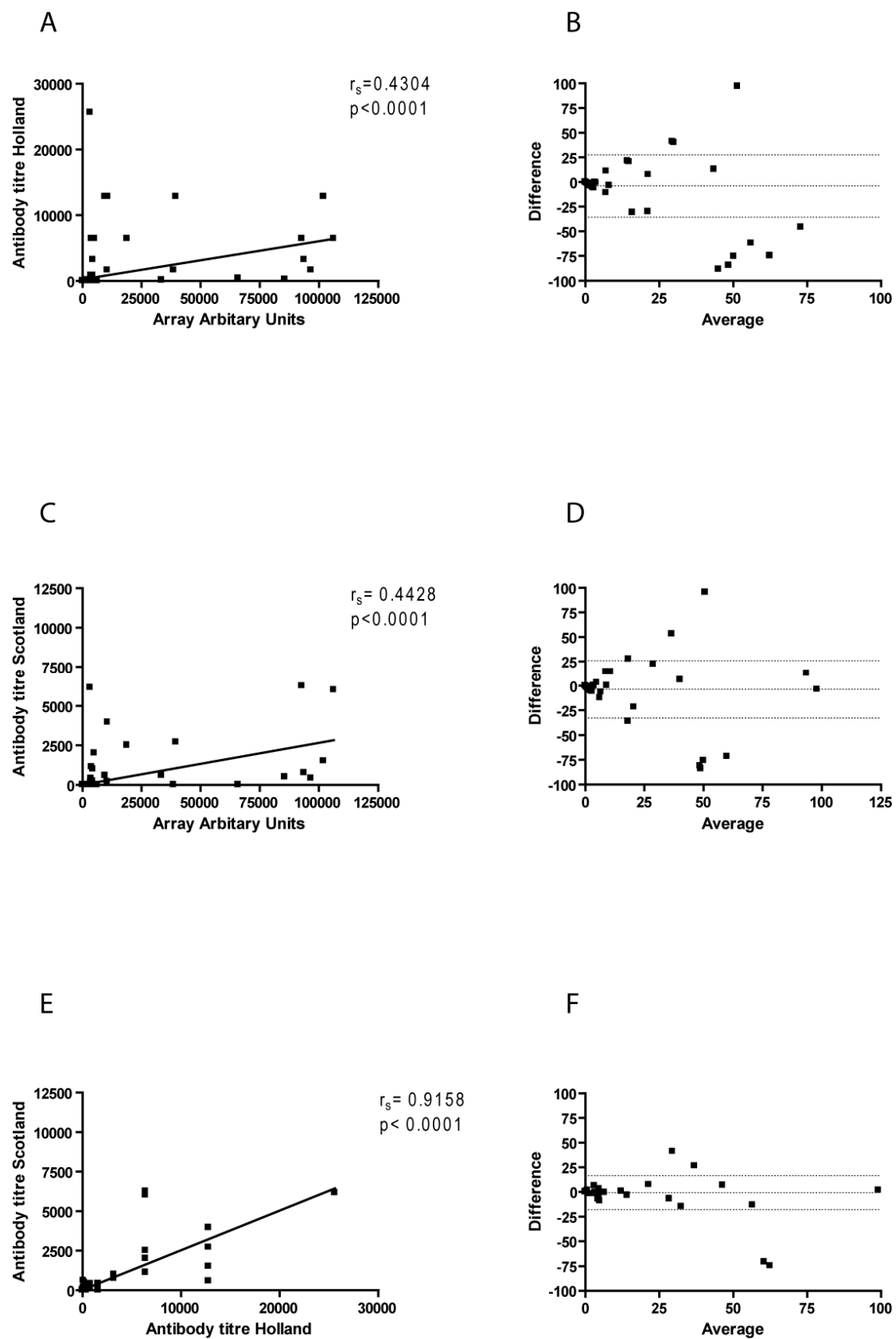


Figure 3.2. Correlation and Bland-Altman graphs for IgG directed against GM1.

$r_s$ , the non parametric Spearman correlation coefficient, has the range -1 to +1. P values quoted are two-tailed p values. The Bland-Altman graphs have three dotted lines intersecting the Y axis, representing the mean difference (middle dotted line) with the dotted line either side of the mean difference representing the 95% limits of agreement.

- A. Correlation of GM1 ELISA performed in Holland with GM1 blot
- B. Bland-Altman graph of GM1 ELISA performed in Holland with GM1 blot
- C. Correlation of GM1 ELISA performed in Scotland with GM1 blot
- D. Bland-Altman graph of GM1 ELISA performed in Scotland with GM1 blot
- E. Correlation of GM1 ELISA performed in Holland and GM1 ELISA performed in Scotland
- F. Bland-Altman graph of GM1 ELISA performed in Holland and GM1 ELISA performed in Scotland



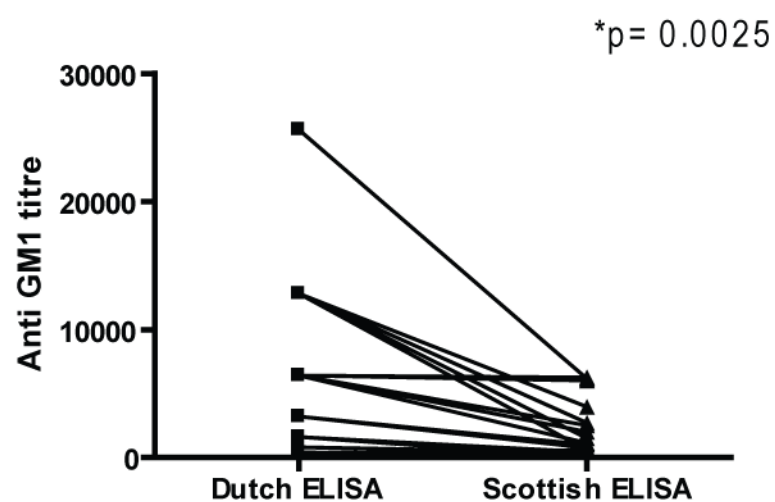


Figure 3.3. Illustrative graph of ELISA titres in AGA GM1 positive patients in Holland and Scotland.

p value quoted is obtained with a 2 tailed t-test.

Table 3.4. Combinatorial binding specificities of sera with detectable IgG to GM1 on ELISA

ID	Dutch ELISA Titre	Scottish ELISA Titre	ELISA OD 490nm	*GM1 ELISA reactivity	GM1 blot intensity	†GM1 blot reactivity	Blot specificity
102	6400	6000	0.77915	positive	106304.68	positive	GM1
116	200	500	0.3394	positive	85609.83	positive	GM1
120	6400	6250	0.9872	positive	92791.63	positive	GM1
140	400	225	0.1654	positive	3743.2	negative	GD1b
152	12800	1500	0.2588	positive	101957.05	positive	GM1
183	6400	1125	0.48335	positive	3916.51	negative	LM1
184	100	0	0	negative	2582.215	negative	GM1:LM1 complex
192	100	600	0.43875	positive	33517.18	positive	GM1
195	800	225	0.18865	positive	4416.245	negative	LM1
222	3200	1000	0.44245	positive	4445.49	negative	sulf:GM1 complex
223	3200	750	0.35825	positive	93685.1	positive	GM1
224	800	400	0.24905	positive	3698.94	negative	sulf:GM1 complex
225	6400	2000	0.4885	positive	5072.735	negative	sulf:GM1 complex
229	400	0	0	negative	65877.9	positive	GM1
230	12800	3950	0.642	positive	10744.135	positive	GM1
233	100	150	0.2511	positive	3645.705	negative	GT1a
241	25600	6150	0.93845	positive	3274.41	negative	GD1a, GT1a
243	1600	425	0.5172	positive	96616.75	positive	GM1
256	6400	2500	0.6045	positive	18898.365	positive	GM1
263	12800	2700	0.9939	positive	39560.935	positive	GM1
292	12800	575	1.0344	positive	9638.715	negative	sulf:GM1 complex
303	1600	0	0	negative	38579.205	positive	GM1
310	1600	0	0.3722	positive	10637.8	positive	GM1
320	100	0	0	negative	2118.285	negative	GD1a

\* ELISA considered to be positive if OD > 0.1

† Blot considered to be positive if average spot intensity > 10000 arbitrary units of detection (obtained using Total Lab software) and associated with a positive spot on visual inspection

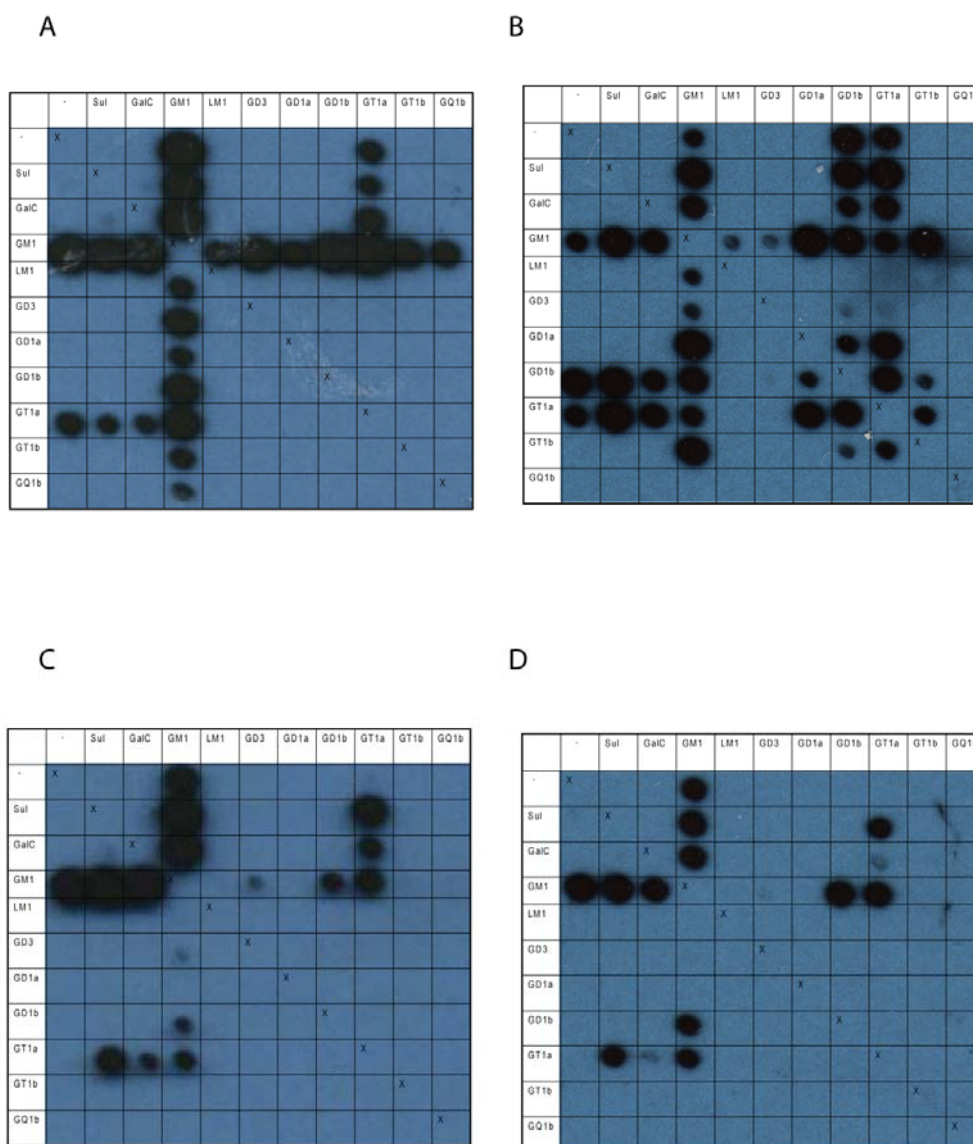
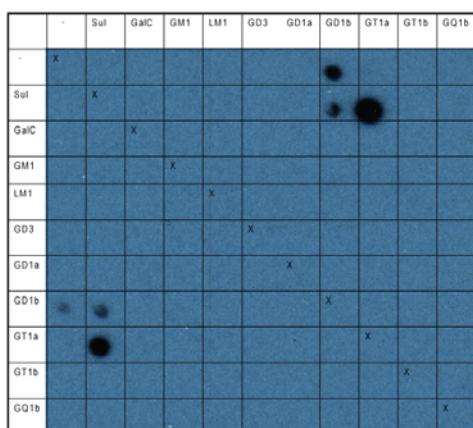


Figure 3.4. Illustrative blots of sera found to be positive for IgG directed against GM1 via both ELISA and combinatorial blot methods.

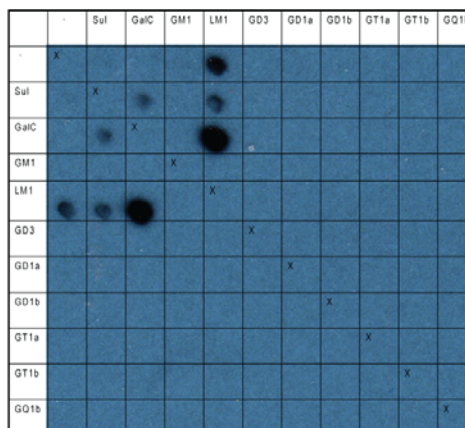
For detailed array methodology see Chapter 2; 2.2.4. Row and column headings reveal the complex at each location, “X”s represent the negative controls (methanol) which act as a line of symmetry for duplicate spots within the same membrane.

- A. Serum 152
- B. Serum 229
- C. Serum 102
- D. Serum 223

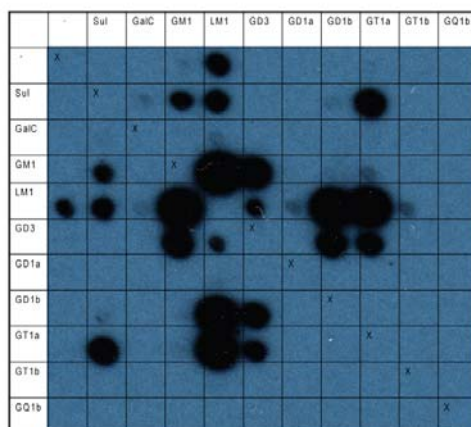
A



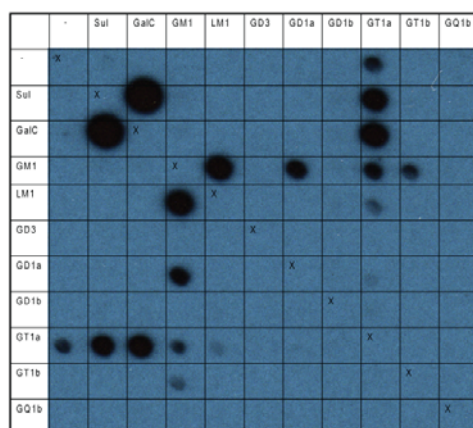
B



C



D



E

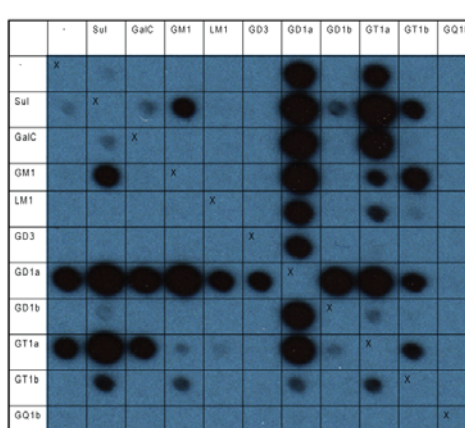
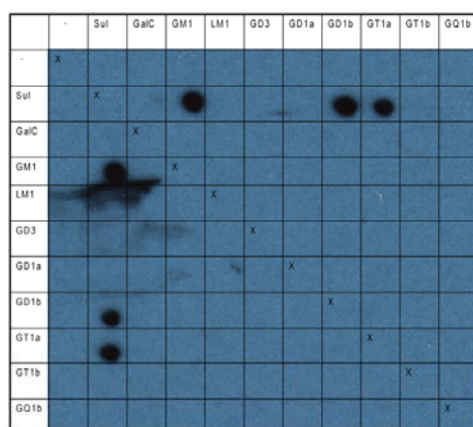


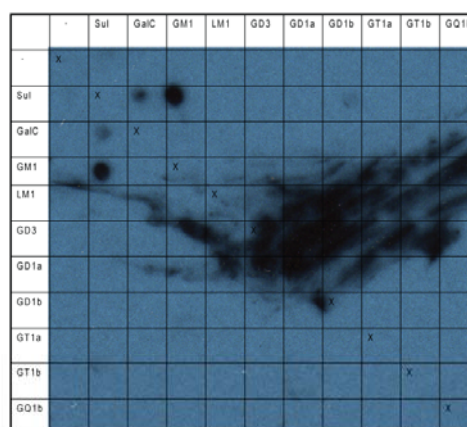
Figure 3.5. Illustrative blots of sera found to be positive on ELISA for GM1 AGA and negative for GM1 AGA on combinatorial blot.

- A. Serum 140 demonstrating binding to GD1b
- B. Serum 183 demonstrating binding to LM1
- C. Serum 195 demonstrating binding to LM1
- D. Serum 233 demonstrating binding to GT1a
- E. Serum 241 demonstrating binding to GD1a & GT1a

A



B



C

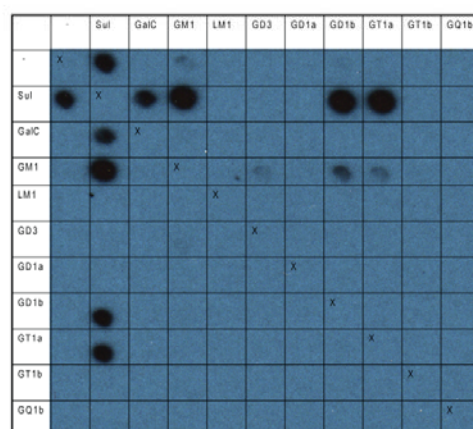


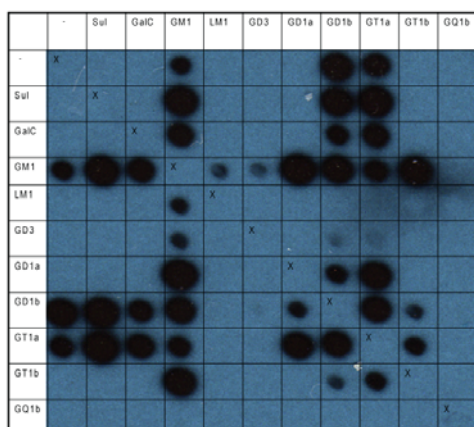
Figure 3.6. Illustrative blots for sera found to be positive on ELISA for GM1 but demonstrated reactivity to complexes on the combinatorial blot

A. Serum 222 demonstrating binding to sulfatide:GM1 complex

B. Serum 224 demonstrating binding to complexes of sulfatide with gangliosides

C. Serum 292 demonstrating binding to sulfatide and complexes of sulfatide with gangliosides

A



B

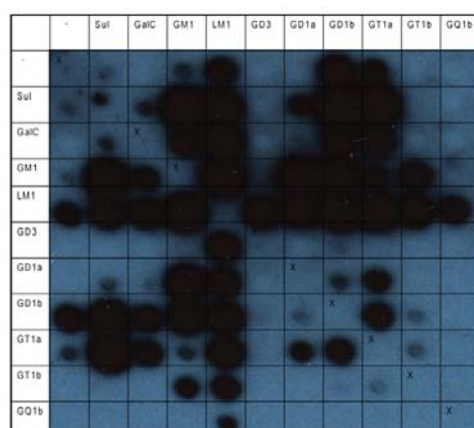


Figure 3.7 Blots of GM1 reactive sera which were negative on ELISA performed in Glasgow

A. Serum 229

B. Serum 303

Our Dutch colleagues had also investigated IgG binding to the gangliosides GD1a and GQ1b. Unfortunately both ODs and titres were unavailable for these assays. However the difference in binding specificities between ELISA and blot observed in GM1 assays was also observed in the GD1a and GQ1b serology (Table 3.5.). 13 serum samples were reported to be positive for GD1a serology by our Dutch colleagues. Only 6 demonstrated IgG reactivity against GD1a using the combinatorial blot method. However a further 6 demonstrated binding to other lipid and lipid complex antigens using the blot method (see Table 3.5.). Illustrative examples are shown in Figure 3.8.

When I performed an ELISA investigating IgG to GD1a in Glasgow using these 13 serum samples, only 7 demonstrated an OD > 0.01. The correlation between the ELISA performed in Glasgow and the combinatorial blot method is depicted in Figure 3.9. (A) ( $r^2=0.5424$ ). Only 1 sample which was positive on ELISA in both Holland and Scotland (OD of 0.139) was negative when investigated using the combinatorial array.

12 serum samples were similarly reported to be positive for GQ1b serology by our Dutch colleagues. Only 2 of these sera demonstrated IgG reactivity against GQ1b using the combinatorial blot method. A further 4 demonstrated binding to other lipid and lipid complex antigens using the blot method (See Table 3.6.). Illustrative examples are shown in Figure 3.10. When I performed an ELISA investigating IgG to GQ1b in Glasgow using these 12 serum samples, only 5 demonstrated an OD > 0.01. The correlation between this ELISA performed in Glasgow and the combinatorial method is depicted in Figure 3.9 (B) ( $r_s= 0.7798$ ).



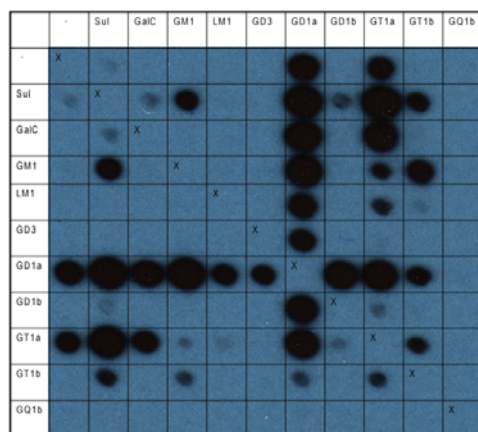
**Table 3.5. Combinatorial binding specificities of sera with detectable IgG to GD1a on Dutch ELISA**

ID	Dutch ELISA	Scottish ELISA OD	*Scottish GD1a ELISA Reactivity	GD1a blot intensity	*GD1a blot intensity	Blot specificity
165	positive	0.139	positive	1512.155	negative	negative
192	positive	0	negative	3184.72	negative	GM1, LM1
230	positive	0	negative	3341.35	negative	GM1
240	positive	0	negative	2836.2	negative	LM1
241	positive	1.37	positive	102831	positive	GD1a
245	positive	0	negative	4047.885	negative	complexes only
279	positive	1.72	positive	110195.9	positive	GD1a, GD1b, GT1b, GQ1b
281	positive	0.46	positive	2867.9	negative	LM1
303	positive	0	negative	12362.01	positive	GM1, LM1, GD1b, GT1a
320	positive	0.88	positive	107914.7	positive	GD1a
332	positive	0	negative	6968.805	negative	complexes only
340	positive	0.1064	positive	19689.23	positive	GD1a, GT1b, GQ1b
345	positive	0.6079	positive	182264	positive	GD1a

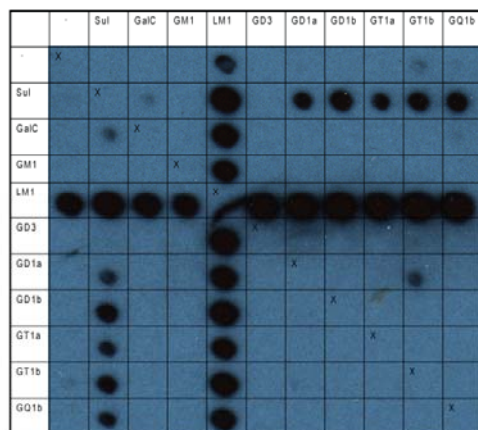
\* ELISA considered to be positive if OD > 0.1

† Blot considered to be positive if average spot intensity > 10000 arbitrary units of detection (obtained using Image Quant Total Lab software) and associated with a positive spot on visual inspection

A



B



C

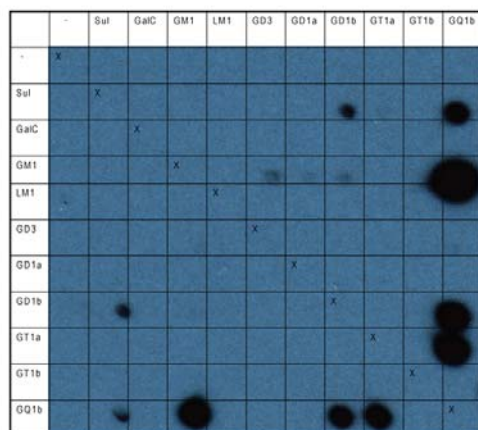


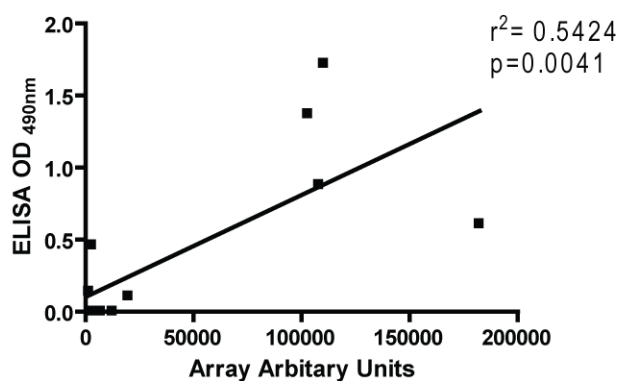
Figure 3.8. Illustrative blots of sera reactive to GD1a on ELISA

A. Serum 241 demonstrating reactivity to GD1a and GT1a

B. Serum 240 demonstrating reactivity to LM1

C. Serum 299 demonstrating reactivity to complexes only

A



B

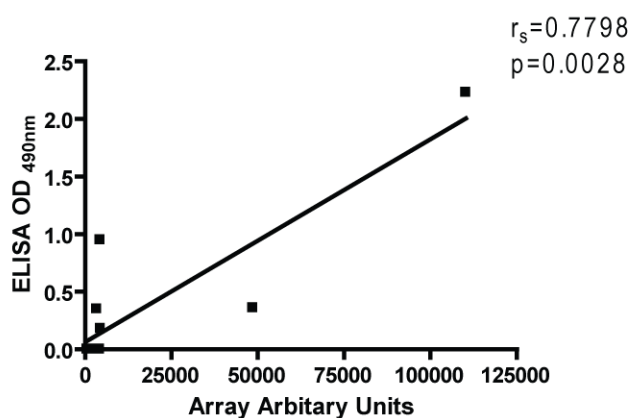


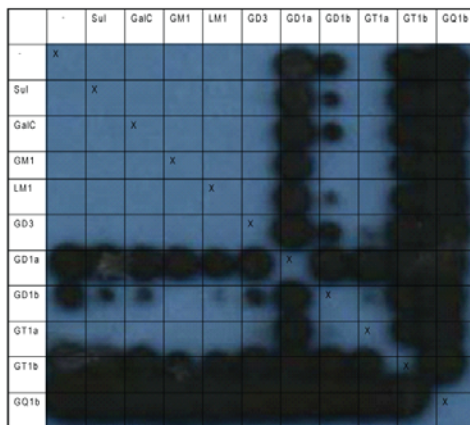
Figure 3.9 Correlation of GD1a and GQ1b IgG in ELISA and combinatorial blot.

P values quoted are two-tailed p values.

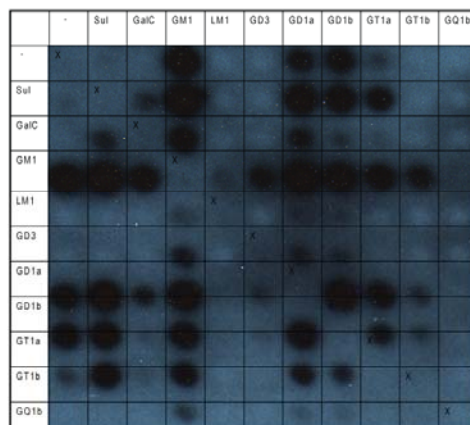
A. IgG against GD1a ( $n=13$ ). As the data is normally distributed the Pearson correlation coefficient is quoted.

B. IgG against GQ1b ( $n=12$ ). As the data is not normally distributed the Spearman rank correlation coefficient is quoted.

A



B



C

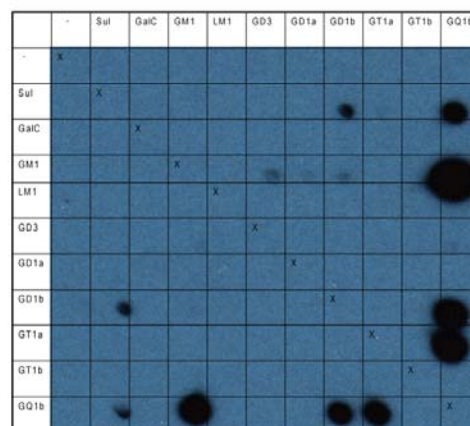


Figure 3.10 Illustrative blots of sera reactive to GQ1b on ELISA

A. Serum 279 demonstrating binding to GD1a, GD1b, GT1b and GQ1b

B. Serum 120 demonstrating binding to GM1, GD1a and GD1b

C. Serum 299 demonstrating binding to complexes only

Table 3.6. Combinatorial binding specificities of sera with detectable IgG to GQ1b

ID	Dutch ELISA	*Scottish ELISA OD	GQ1b ELISA reactivity	GQ1b blot Intensity	†GD1a blot reactivity	Blot specificity
108	positive	0	negative	614.52	negative	negative
120	positive	0	negative	2170.895	negative	GM1, GD1a, GD1b, GT1b
165	positive	0	negative	1783.585	negative	negative
240	positive	0.35	positive	3372.45	negative	LM1
253	positive	0.18	positive	4451.205	negative	GD1a, GT1a
257	positive	0	negative	3083.29	negative	negative
267	positive	0	negative	3535.99	negative	negative
279	positive	2.23	positive	110231.9	positive	GD1a, GD1b, GT1b, GQ1b
299	positive	0.95	positive	4318.79	negative	complexes only
319	positive	0	negative	4167.6	negative	negative
340	positive	0.36	positive	48570.29	positive	GD1a, GT1b, GQ1b
355	positive	0	negative	1020.515	negative	negative

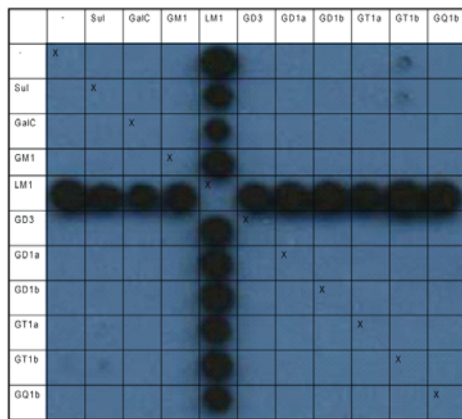
\* ELISA considered to be positive if OD > 0.1

† Blot considered to be positive if average spot intensity > 10000 arbitrary units of detection (obtained using Total Lab software) and associated with a positive spot on visual inspection

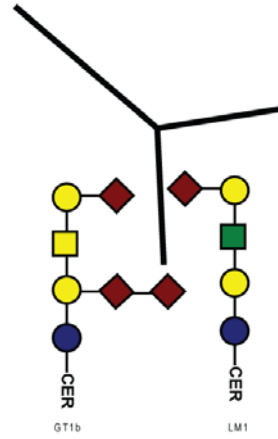
Using the combinatorial array we identified that 42/180 GBS samples demonstrated IgG binding to glycolipid targets. Within these 42 samples which demonstrate IgG binding on the combinatorial arrays three patterns of binding emerge.

The first (see Figure 3.11. (A)) is described as complex independent. Here antibody binds to the lipid antigen when presented singly and when presented in combination with other lipids. The second is complex enhanced (Figure 3.11. (B)) with increased/specific binding demonstrated to lipid antigens when complexed together on the membrane and was observed in 16/42 seropositive patients. In keeping with Kaida's definition of anti-ganglioside complex antibodies I defined complex enhanced binding as being present if the intensity for the IgG directed against the lipid complex was more than the sum of the intensities against each contributing lipid (Kaida et al. 2007). The third pattern of binding occurs when anti-glycolipid antibody binding is attenuated when the lipid antigen is partnered with specific lipids (Figure 3.11 (C). However these patterns are an oversimplification as many sera would demonstrate more than one pattern on the array (see Figure 3.4 (A) & (D).

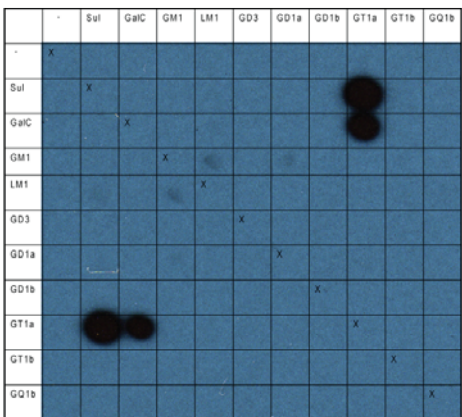
A



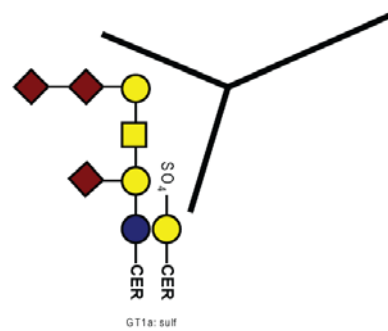
B



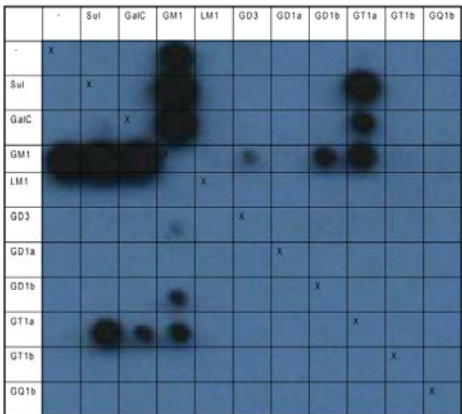
C



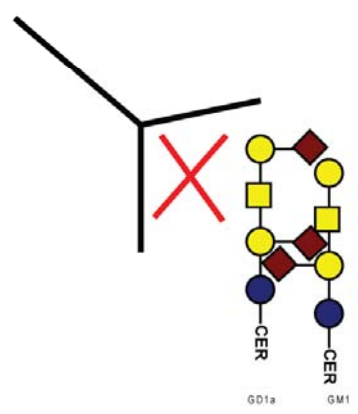
D



E



F



GalNAc



NeuAc



GluNAc



Gal



Glc

Figure of 3.11 Three patterns of binding to glycolipid antigens

Key:

GalNAc = *N*-Acetylgalactosamine

NeuAc= *N*-Acetylneuraminic acid

GluNAc= *N*- Acetylglucosamine

Gal= Galactose

Glc= Glucose

Cer= ceramide

- A. Serum 281 with complex independent pattern of antibody binding to the lipid LM1
- B. Cartoon of complex independent antibody binding to LM1
- C. Serum 245 with antibody binding only to the complex created by GT1a and either sulfatide or galactocerebroside.
- D. Cartoon demonstrating complex dependent binding to GT1a:sulfatide complex
- E. Serum 102 demonstrating inhibition of anti GM1 IgG binding when GM1 is complexed with particular lipids e.g. LM1, GD1a, GT1b and GQ1b
- F. Cartoon demonstrating inhibition of antibody binding to GM1 when GM1 is complexed with GD1a



Using the combinatorial array allows us to identify that 42/180 GBS samples demonstrate IgG binding to glycolipid targets (Figure 3.12.). 16 of these bound only to glycolipids presented either singly or as part of a complex with another lipid. A further 11 bound lipid in this manner but in addition bound “new” complexes of lipids. Within both groups it was observed that there was enhancement of binding to complexes of lipids. This “complex reactivity” has been defined as being present when the sum of the blot intensity of IgG directed against a pair of lipids is more than the sum of the IgG blot intensity directed against each individual contributory lipid. 6/16 sera binding to single glycolipids and their complexes demonstrated enhanced binding to complexes. 5/11 sera which bound single glycolipids and new complexes also demonstrated increased binding to specific combinations of lipids compared to the intensity observed for each lipid partner. Therefore 11 sera which demonstrated IgG binding to single lipids demonstrated anti-GSC antibodies using Kaida’s definition.

If we employ a stricter definition of complex reactivity in which there is no discernible binding to each contributory lipid partner in the complex it can be observed that there are 15 patients demonstrating this pattern. The binding specificity of these sera is outlined in Table 3.7.

As outlined earlier 39/180 samples had been assayed using ELISA by our Dutch colleagues. 6 of these samples proved to be negative using the combinatorial method. The combinatorial method allowed the detection of a further 9 sera which bound predominantly to complexes of glycolipids rather than to single glycolipids (see Table 3.8.). None of the healthy control sera demonstrated any IgG binding to glycolipids or their complexes using the combinatorial array.

The sera which were positive on the combinatorial array represented 10 patients with neurophysiology findings in keeping with AIDP and 3 patients with AMAN. The rest of these anti-glycolipid positive patients had equivocal findings ( $n=22$ ) or unavailable results ( $n=7$ ). These glycolipid reactive patients were more likely to have suffered from diarrhoea ( $\chi^2$   $p=0.0033$ )

and have proven serology for campylobacter jejuni ( $\chi^2$   $p < 0.0001$ ) compared to those patients who tested negative on the array. There was no difference between the two groups of patients in the history for a preceding upper respiratory tract infection or serology for mycoplasma and cytomegalovirus (CMV).

Patients who did not demonstrate any glycolipid binding on the array were more likely to have pain ( $\chi^2$   $p = 0.0011$ ), paraesthesiae ( $\chi^2$   $p = 0.0028$ ) and a cranial nerve deficit ( $\chi^2$   $p = 0.0022$ ). Both antibody positive and negative groups were just as likely to require ventilation.

Interestingly patients who demonstrated binding of any nature to glycolipids antigens on the combinatorial array ( $n=42$ ) had significantly lower MRC-sum scores indicative of more severe disability at presentation ( $p=0.0001$ , Mann Whitney, see Figure 3.13 (A)). This difference in severity was still present though less marked at week 3 ( $p=0.0195$ , Mann Whitney, see Figure 3.13 (B)). Those patients with complex reactivity ( $n=26$ ) did not differ in MRC scores from patients with reactivity to single glycolipids antigens (see Figure 3.13 (C) & (D)).

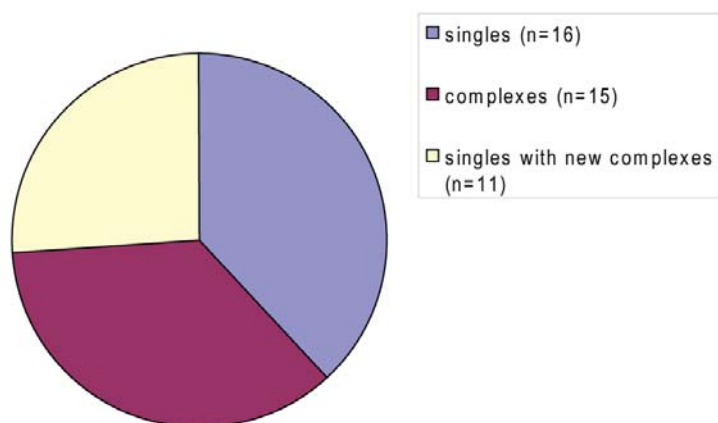
Table 3.7. Sera demonstrating IgG only to complexes of lipids with no demonstrable binding to individual glycolipids

ID	Glycolipid complex
101	Sulfatide in complex with GM1/GD1b/GT1a
104	Sulfatide in complex with GM1/GD1b/GT1a
135	LM1 in complex with GM1/GD1b/GT1a Galactocerebroside in complex with GT1a
184	LM1 in complex with GM1/GD1b/GT1a Sulfatide in complex with galactocerebroside/GT1a
191	Sulfatide in complex with GM1/GT1a
222	Sulfatide in complex with GM1/GD1b/GT1a
224	Sulfatide in complex with galactocerebroside/GM1
225	Sulfatide in complex with GalC/ GM1/GD1b/GT1a
242	Galactocerebroside:GT1b, GM1:GD1a
244	GT1a in complex with sulfatide/galactocerebroside
245	GT1a in complex with sulfatide/galactocerebroside
299	GQ1b in complex with sulfatide/GM1/GD1b/GT1a
304	Sulfatide in complex with galactocerebroside/LM1
332	Sulfatide in complex with LM1/GD1a/GD1b/GT1a/GT1b & LM1 in complex with GD3/GD1b/GT1b
337	Sulfatide in complex with GM1/GD1b/GT1a Galactocerebroside in complex with GM1/GT1a GM1 in complex with LM1/GD1a/GD1b

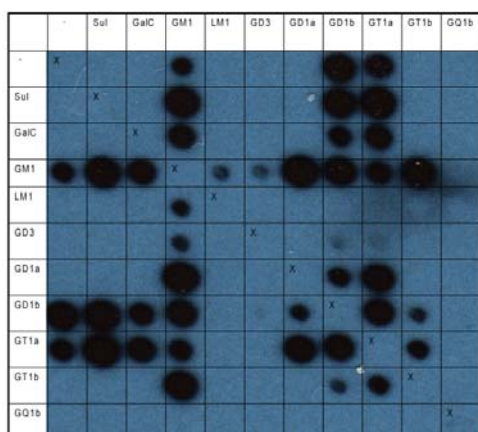
Table 3.8. Additional sera identified using the combinatorial array

ID	Complexes only?	Combinatorial array specificity
101	Yes	Sulfatide in complex with GM1/GD1b/GT1a
104	Yes	Sulfatide in complex with GM1/GD1b/GT1a
135	Yes	GM1 in complex with LM1/GD1b/GT1a
191	Yes	Sulfatide in complex with GM1/GT1a
242	Yes	GalC:GT1b complex, GM1:GD1a complex
244	Yes	GT1a in complex with sulfatide/galactocerebroside
265	No	GT1a, and GM1/LM1 complex
304	Yes	Sulfatide:GM1 complex
337	Yes	Sulfatide:GM1 complex

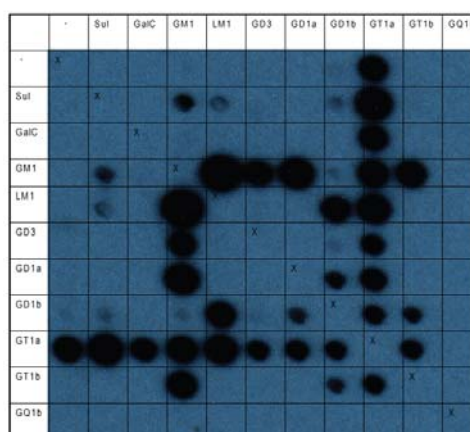
A



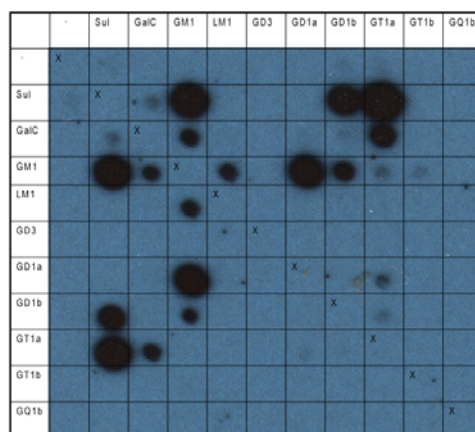
B



C



D



**Figure 3.12. Breakdown of sera positive using combinatorial blot**

- A. Pie chart demonstrating IgG binding pattern of 42/180 positive sera
- B. Illustrative blot from serum 229 demonstrating binding to GM1, GD1b and GT1a
- C. Illustrative blot from serum 265 demonstrating binding to GT1a and to the complex created by GM1:LM1
- D. Illustrative blot from serum 337 demonstrating binding to complexes only

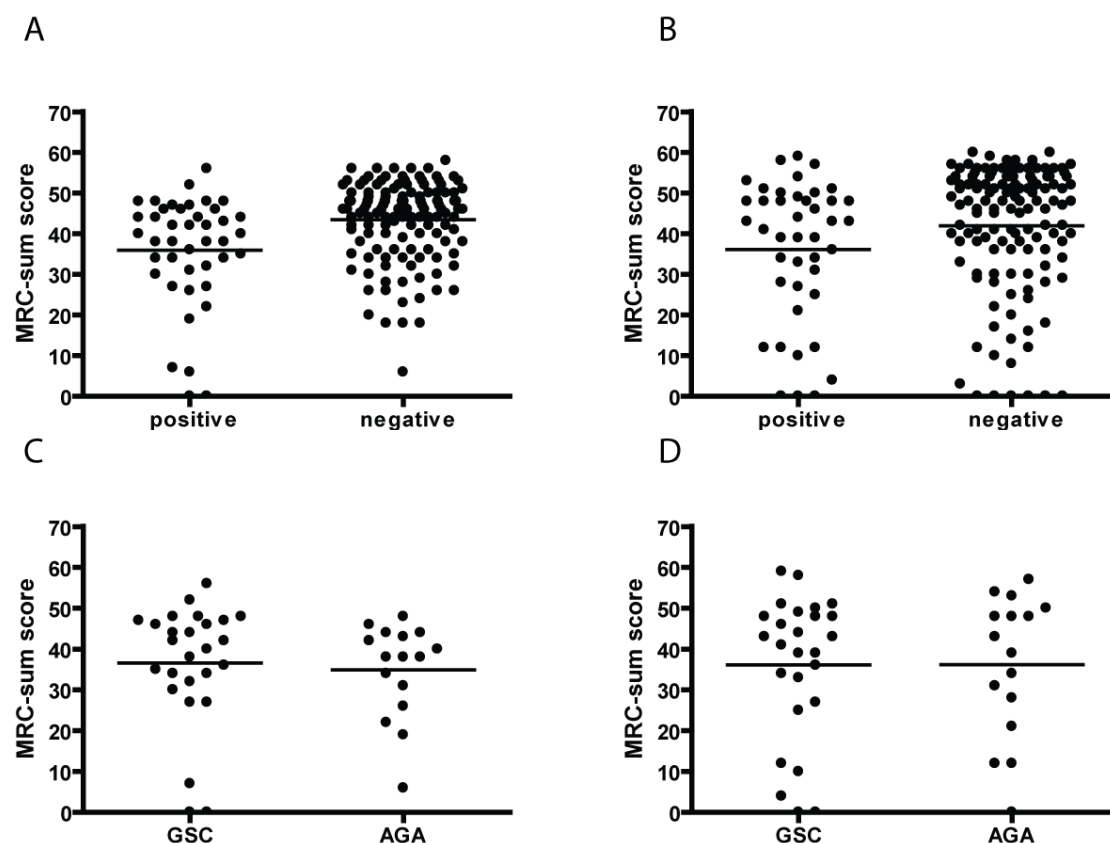


Figure 3.13. MRC-sum scores in glycolipid reactive and unreactive patients

Severity of weakness is expressed as MRC-sum scores- this is a sum of scores according to the Medical Research Council grading system of 6 bilateral muscle groups ranging from 60 (normal strength) to 0 (tetraparalytic)

A. MRC-sum scores in AGA positive (n=42) and negative (n=138) patients at presentation

B. MRC-sum scores in AGA positive (n=42) and negative (n=138) patients at week 3

C. MRC-sum scores in GSC positive (n=26) and AGA (to single lipid antigens only) (n=16) patients at presentation

D. MRC-sum scores in GSC positive (n=26) and AGA (to single lipid antigens only) (n=16) patients at week 3

Different measures of hierarchical clustering were applied to the data which was logarithmically transformed (log 2). This divided the 42 glycolipid reactive sera into subgroups depending on their glycolipid antibodies profile. Covariance clustering ordered the dataset into 4 separate groups with different ganglioside and clinical phenotypes (Figure 3.15 labelled 1-4).

10/12 sera from Group 1 demonstrated IgG reactivity to LM1 either in isolation or in complex with other lipids. This group were more likely to have reactivity to the GM1/LM1 complex compared to the rest of the glycolipid positive group. 6 of these 12 sera demonstrated anti-GM1/LM1 complex reactivity (defined as a blot intensity for complex of lipids > sum of the blot intensity of the two contributory lipids) compared to 2/30 ( $p=0.0036$ , 2 tailed Fishers exact test).

All 12 sera comprising Group 2 demonstrated IgG reactivity to GM1 either presented in isolation or in combination with other lipids such as sulfatide. 11/12 demonstrated binding to GM1 in isolation. This is in direct contrast to the glycolipid antibody profile of Group 1 (Fishers exact test  $p=0.0001$ ), Group 3 (Fishers exact test  $p=0.0006$ ) and group 4 ( $p=0.0004$ ). When compared to all other samples  $p < 0.0001$ .

All 12 sera comprising group 3 demonstrated IgG reactivity to the complex created by sulfatide and GT1a again using the same definition as before. This again is in contrast to the rest of the antibody positive cohort (Fishers exact test,  $p=0.0003$ ). Group 4 comprised sera which bound GD1a in isolation and when complexed with other lipids. 5/6 sera in this group bound the single lipid GD1a compared to 1/35 remaining glycolipid reactive group (Fishers exact test,  $p<0.0001$ ).



In addition to clustering ordering the dataset into different groups with distinct ganglioside antibody profiles, these groups have different clinical phenotypes. Group 1 were more likely to have neurophysiology pertaining to the AIDP variant compared to Group 2 ( $p = 0.0373$ , Fishers exact test). Group 1 patients were also more likely to have a sensory deficit at week 4 of the illness, compared to Group 2 ( $p = 0.0123$ ) and, indeed, all other antibody positive patients ( $p = 0.0061$ ). Group 1 patients were least likely to have suffered from a preceding *Campylobacter jejuni* infection compared to the other three groups ( $p = 0.04$ , Fishers exact test).

Groups 3 & 4 were more likely to suffer from pain as opposed to Groups 1 & 2 ( $p = 0.0188$ , Fishers exact test). Group 4 were more likely to have a cranial nerve deficit compared to Groups 2 & 3 ( $p = 0.0021$ , Fishers exact test).

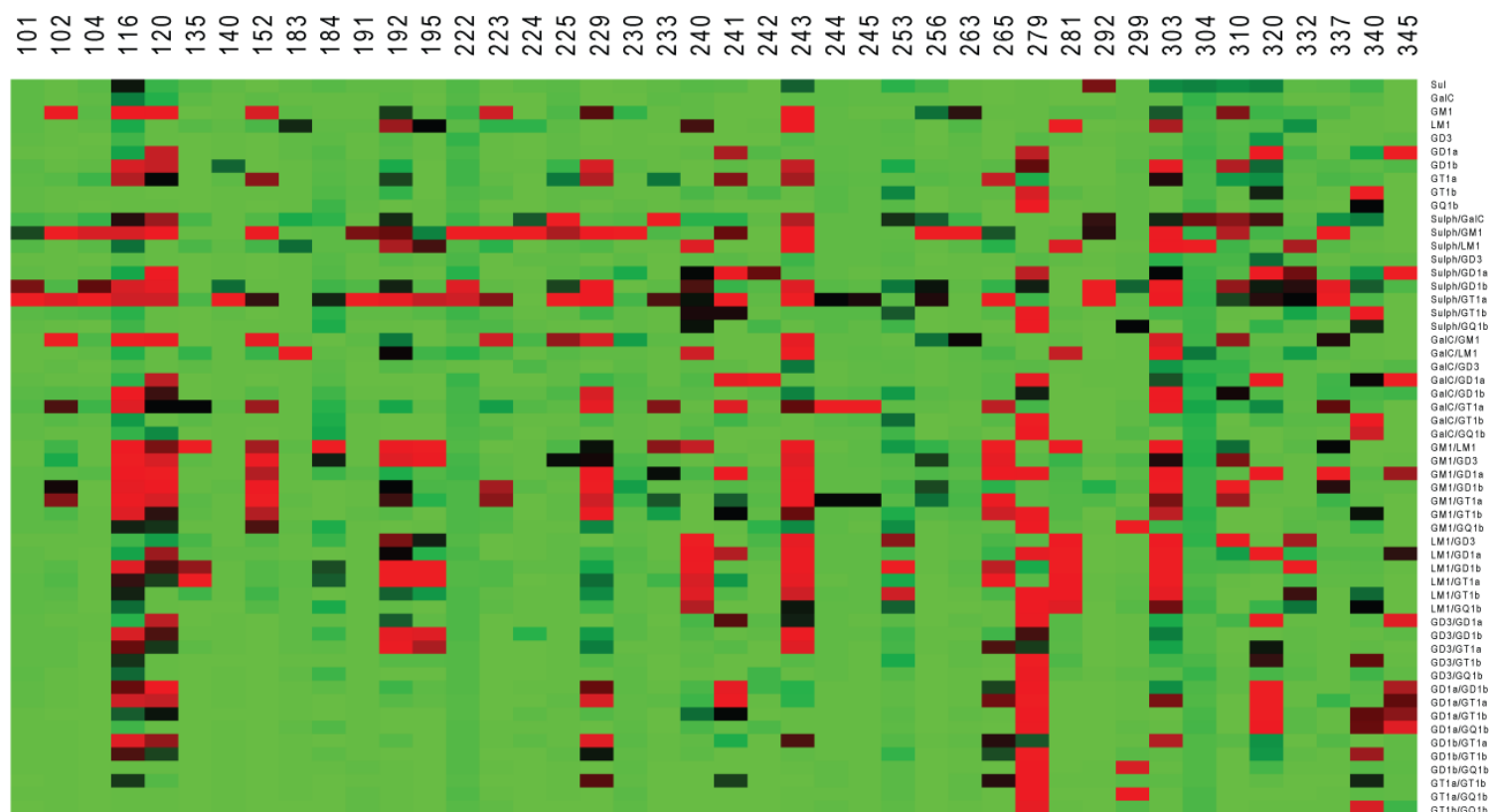


Figure 3.14. Heatmap of 42 glycolipid reactive sera  
Unclustered heatmap depicts reactivity of sera ( $n=42$ ) which bind to glycolipids using the combinatorial array. Each individual serum is represented by each column with each row representing lipid antigens.

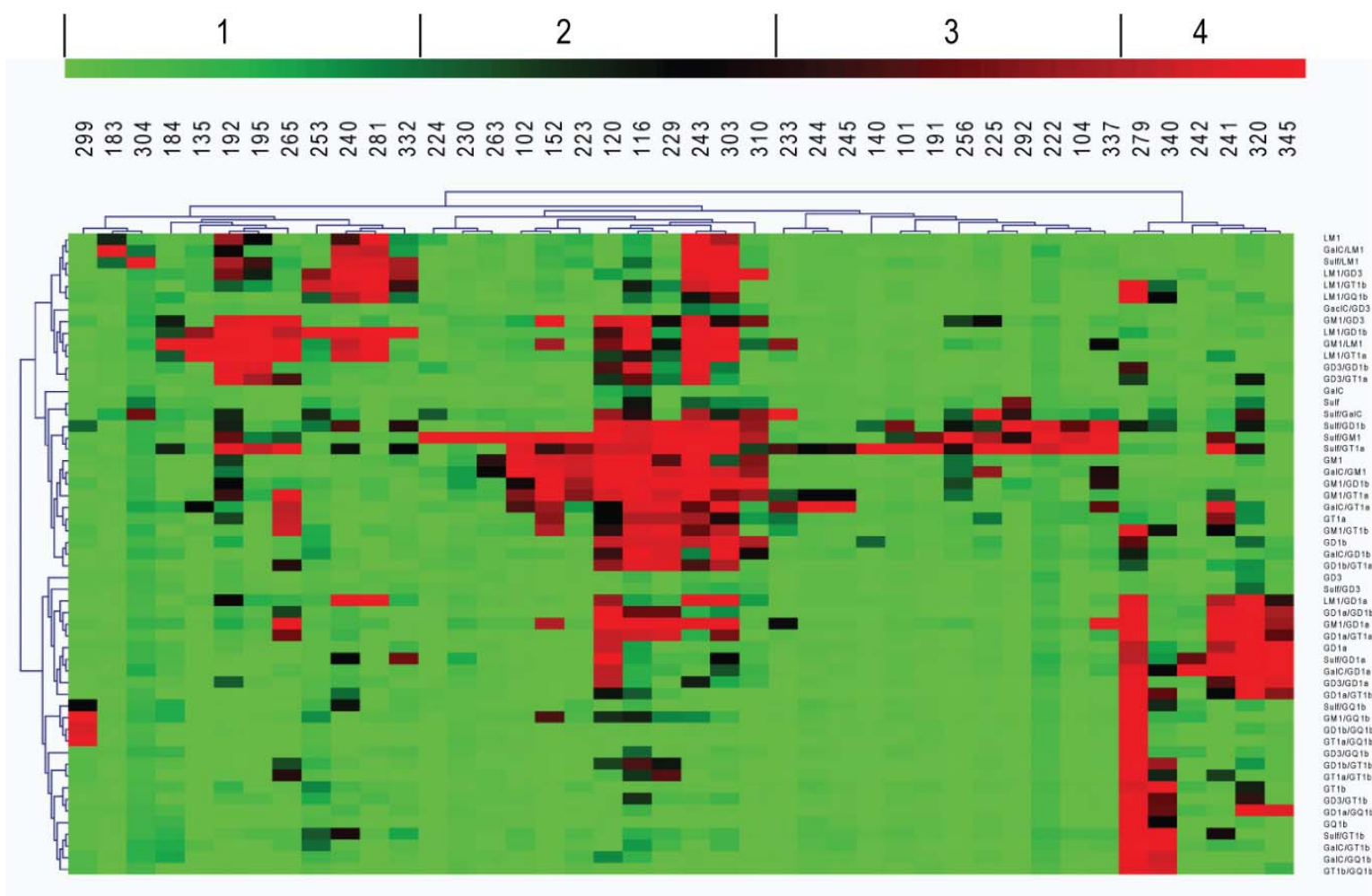


Figure 3.15. Covariance clustering of heatmap

Covariance clustering has been performed revealing 4 separate groups with different glycolipid and clinical phenotypes.

I was interested to observe that the 12 sera comprising group 3 demonstrated IgG reactivity to the complex created by sulfatide and GT1a. I then investigated the responses to sulfatide, GT1a and the complex of sulfatide and GT1a using ELISA in 7 of these samples (see Figure 3.16.). The findings on ELISA were similar to those observed using the combinatorial array technique in that binding to the complexes of sulfatide:GT1a was enhanced. In 5 of these samples enhanced binding was also evident to the complex created by sulfatide and GM1 (see Figure 3.16.(C)).

I next performed a TLC of the individual glycolipids to assess lipid purity. As illustrated in Figure 3.17 single spots can be observed for the glycolipids GM1, sulfatide and GD1b. However the GT1a preparation had multiple spots indicating possible contamination with other lipids.

However I needed to further investigate whether these complex reactive sera were truly binding a complex of GT1a and sulfatide or another lipid and sulfatide. To do this I performed a TLC of GT1a (as before). After the GT1a separated, I then printed a line of sulfatide across the migration path of the GT1a and then probed with sera or the monoclonal antibody (DG2) (Figure 3.18). DG2, a mouse monoclonal antibody, recognizes the terminal Gal-GalNAc of GM1. Therefore it should not bind GT1a- however it binds to GT1a on the combinatorial array using DG2 (see Figure 3.19). Using TLC however it is evident that the DG2 is binding to GM1 which is part of the GT1a/ lipid mix. The serum 104 is shown alongside DG2. It can be observed that there appears to be three localised areas of binding with one corresponding to GM1 and the other 2 representing unknown lipid fractions. Of note there was no demonstrable binding to the GT1a fragment using this technique.

Further mass spec analysis performed on the lipid solution of GT1a by Dr Richard Goodwin of the University of Glasgow confirmed that GT1a was contaminated by large amounts of GM1 which may explain why the monospecific anti-GM1 antibody bound GT1 in addition. The contamination of the GT1a may reflect the presence of a sialidase which have cleaved sialic acid residues from GT1a to create GM1. The next step is to identify

these lipids by using mass spectrometry of the individual lipid fractions from the TLC plates.

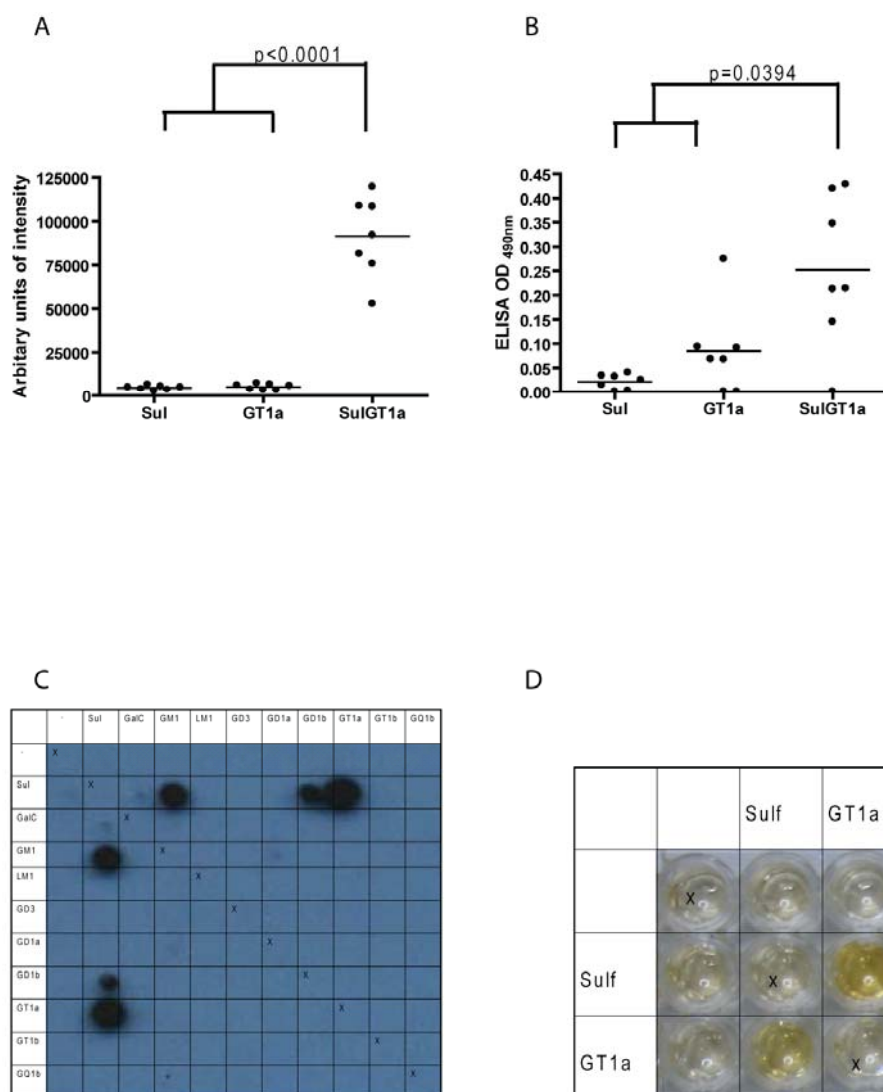


Figure 3.16. Anti sulfatide:GT1a complex antibodies

A. Intensities for IgG against sulfatide, GT1a and sulfatide GT1a in 7 patients. Binding to the complex of sulfatide and GT1a is enhanced compared to that of the sum of the intensities for the individual lipids (paired t test,  $p < 0.0001$ )

B. Findings on ELISA for the same 7 patients. Binding to the complex of sulfatide and GT1a is enhanced compared to that of the sum of the intensities for the individual lipids (paired t test,  $p = 0.0394$ )

C. Illustrative blot from serum 104

D. Illustrative ELISA from serum 104

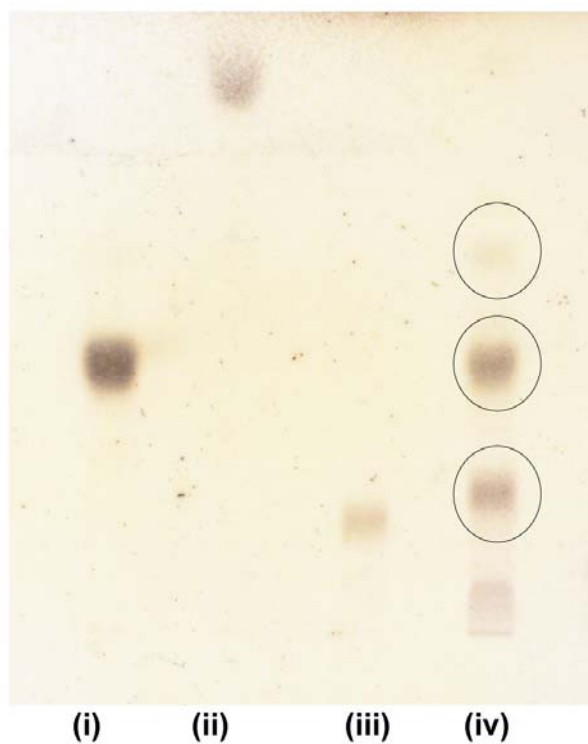


Figure 3.17. TLC of glycolipids

- (i) GM1
- (ii) Sulfatide
- (iii) GD1b
- (iv) GT1a

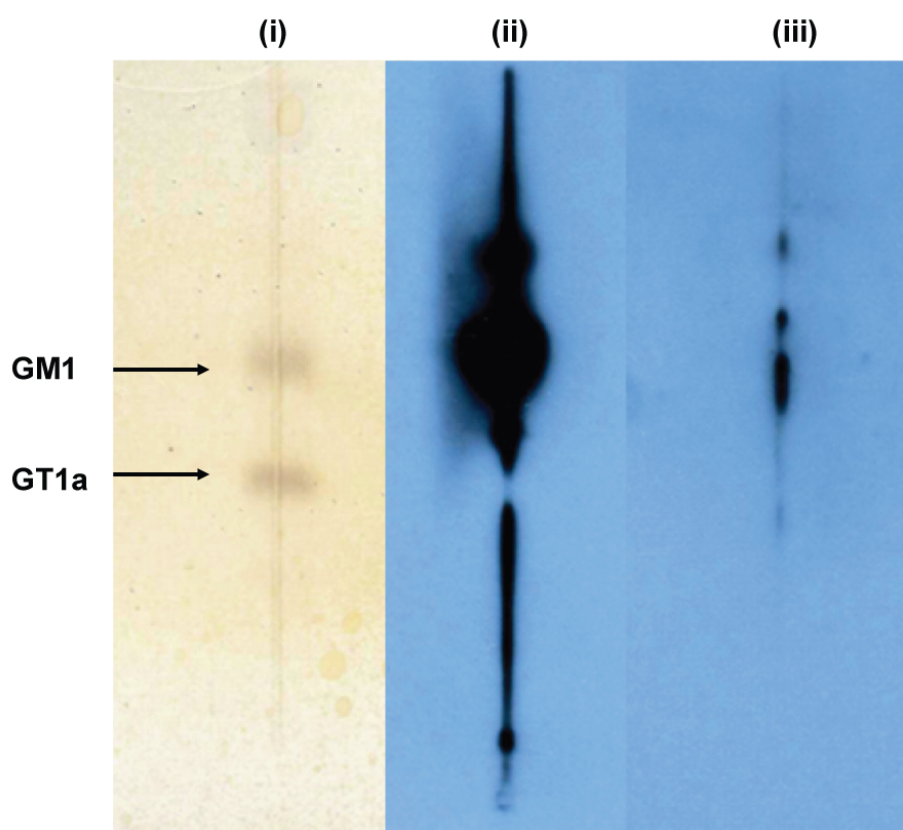


Figure 3.18. TLC overlay of GT1a + sulfatide

TLC of GT1a performed as before. After the TLC plate was removed from the running buffer a line of sulfatide was then sprayed across the TLC plate intersecting the GT1a.

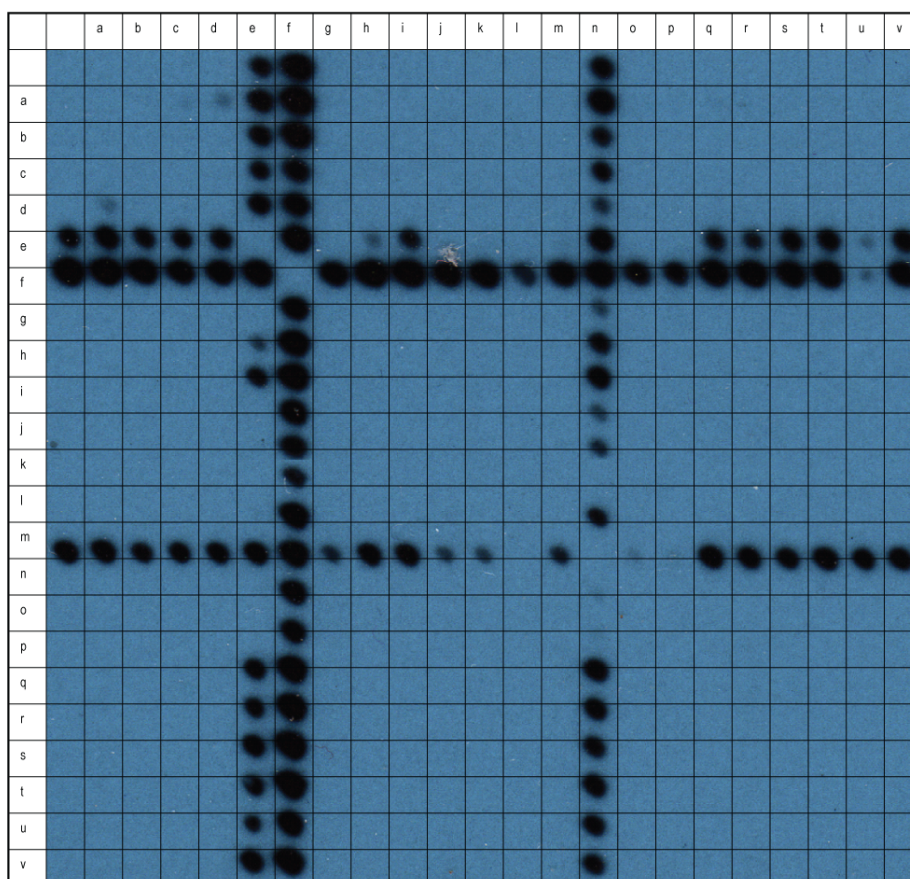
(i) GT1a separated using TLC and stained with resorcinol

(ii) TLC overlay with the monoclonal anti-GM1 antibody GM1 DG2

(iii) TLC overlay with serum 104



A



B

a	Sulfatide	l	GD1a
b	Galactocerebroside	m	GD1b
c	Cholesterol	n	GT1a
d	Sphingomyelin	o	GT1b
e	GA1	p	GQ1b
f	GM1	q	DGG
g	GM2	r	L alpha Phosphatidylcholine
h	GM3	s	L alphaphosphatidylethanolamine
i	GM4	t	L alpha Phosphatidylinositol-4-phosphate
j	GD2	u	Plasmalogen
k	GD3	v	L alphaphosphatidylserine

Figure 3.19. DG2 array combinatorial array specificity

A. Illustrative blot of DG2 on combinatorial array demonstrating binding to GA1, GM1 and GT1a

B. Key to combinatorial array

### 3.4. Discussion

42/180 (23.3%) of GBS patients demonstrated evidence of IgG binding to glycolipids or their complexes using the combinatorial array. A wide variety of glycolipids and their complexes were bound by these sera. Seropositive patients represented patients with AIDP (n=10) and AMAN (n=3) however a large number had equivocal findings on neurophysiological testing (n=22) with data unavailable for 7 patients. Seropositive patients were distinct from seronegative patients in that they were more likely to have suffered from diarrhoea and had a *campylobacter jejuni* infection and were less likely to suffer from pain, paraesthesiae and a cranial nerve deficit. Seropositive (9/42) and seronegative (32/138) patients were equally at risk of the need for ventilation and both groups had similar rates of infection with mycoplasma and CMV. On initial assessment those patients with glycolipids antibodies were more likely to have lower MRC-sum scores indicative of greater disability however by week 3 of the illness MRC-sum scores were similar between the anti-glycolipid positive and negative groups.

Within the glycolipid reactive cohort with the aid of clustering techniques it was possible to separate this cohort of 42 into 4 groups with different glycolipid binding profiles. Group 1 (n=12) represented sera from patients predominantly with the AIDP phenotype.

AIDP was initially thought to result from T-cell dependent mechanisms as lymphocytic inflammation has been described on nerve biopsies (Asbury et al. 1969). However more recent work involving pathology of autopsy cases suggests that antibody dependent mechanisms are involved (Hafer-Macko et al. 1996). The pathology described closely resembled that seen in experimental autoimmune neuritis induced by galactocerebroside antibody (K. Saida et al. 1979) (T. Saida et al. 1979). Galactocerebroside has been investigated as a possible antigen in a number of GBS series with a

number of these studies suggesting a relationship between *mycoplasma pneumoniae* infection and the development of anti-galactocerebroside antibodies (Hughes et al. 1984) (Kusunoki et al. 1995) (Hao et al. 1998). But the observed numbers of galactocerebroside sera are small for example Kusunoki's data describes 4/82 GBS patients having galactocerebroside antibodies. The relevant neurophysiological data was not available for all patients although Kusunoki did report that 2 of these 4 patients had neurophysiological findings supportive of AIDP. Amongst many other lipids my study has investigated the IgG response to galactocerebroside in 180 GBS patients of whom 56 have neurophysiological findings in keeping with AIDP. No patient in this entire cohort demonstrated binding to galactocerebroside which would appear to suggest that this is not an important auto antigen in GBS.

However this particular group (Group 1) of patients which had neurophysiology pertaining to AIDP, displayed a specific binding profile on the combinatorial array, namely to LM1 and complexes of LM1 with the closely related glycolipid GM1. LM1 is considered to be a major peripheral nerve myelin glycolipid with a structure closely related to that of GM1 however the LM1, the N-acetylgalactosamine molecule is replaced with N-acetylglucosamine (Ogawa-Goto et al. 1998). Certainly there does appear to be stronger evidence for an association between LM1 antibodies and GBS than for galactocerebroside. In 1992 Ilyas et al demonstrated that 11/53 GBS patients had anti-LM1 antibodies (Ilyas, Mithen, Dalakas, Chen & Cook 1992a). However an earlier study had reported anti-LM1 antibodies in 30% of their normal controls. A later study appeared to suggest an association between IgG LM1 antibodies and AIDP with Yako et al reporting, after screening of a large cohort of patients ( $n=140$ ), that 5/7 patients with detectable IgG to LM1 had neurophysiology in keeping with AIDP (Yako et al. 1999). However further studies were at odds with this finding reporting the presence of LM1 antibodies in AMAN and AMSAN ( $n=8$ ) in addition to 1 AIDP patient (Susuki et al. 2002). Interestingly sera from the axonal patients

demonstrated a polyspecific pattern of glycolipid antibody binding with IgG binding a variety of gangliosides however the IgG from the AIDP patient was monospecific for LM1. Only one of the LM1 reactive sera in my cohort was monospecific for LM1 and LM1 complexes (see Figure 3.11. (A)). This particular patient had equivocal findings on neurophysiological testing. The rest of the Group 1 sera were “polyreactive” but reacted significantly more to complexes of LM1 with GM1 compared to the rest of the seropositive sera.

Group 2 ( $n=12$ ) represented sera which bound GM1. These patients were less likely to have AIDP and probably represented patients with AMAN. Data from the TLC experiments would suggest that Group 3 may, in fact, share more in common with group 2. We are limited however in profiling these patients accurately due to the high number of patients with equivocal and unexcitable neurophysiology (89/180).

Another lipid of potential importance is SGPG (sulfated glucuronidated paragloboside). SGPG has a similar structure to LM1, except for a 3-sulfated glucuronic acid instead of a sialic acid on the terminal saccharide chain. SGPG shares carbohydrate antigenic moieties with myelin associated glycoprotein (MAG). Furthermore around 60% of patients with IgM paraproteinaemic neuropathy will demonstrate binding to SGPG (Ilyas et al. 1991). A number of investigators have looked at the IgG response to SGPG in GBS. 5/53 patients with GBS were reported to have antibodies to this glycolipid however a further larger study failed to detect anti-SGPG IgG in a cohort of 96 (Ilyas et al. 1991) (Yuki, Tagawa & Handa 1996a). It would be interesting to include this lipid on future combinatorial arrays.

The position of glycolipids in functionally distinct membrane microdomains, termed function rafts, is likely to be relevant in the binding capabilities of anti glycolipid antibodies (Simons et al. 1997). This has been demonstrated aptly by Greenshields et al (Greenshields et al. 2009). The investigators revealed that the local environment directly influenced the binding capabilities of monoclonal anti glycolipid antibodies. Two monoclonal GM1 antibodies were investigated, termed DG1 and DG2. These antibodies were generated by immunisation of GalNAC-transferase deficient mice with GM1 preparations. This breed of mice lack all ganglioside more complex than GM3/GM3. When immunized with GM1 these mice generate IgG against this lipid because of an inherent lack of immunological tolerance. However only one of these antibodies, DG2, demonstrated binding to ex-vivo motor nerve terminals. As a result of DG2 antibody binding, complement mediated damage ensued. DG1 and DG2 antibodies had similar affinities for GM1 in isolation however when GM1 was complexed with other lipids the binding of the DG1 antibody was significantly attenuated presumably thereby rendering the DG1 antibody pathologically inert. This data was obtained using the combinatorial array methodology I developed. This difference in binding specificity on the combinatorial array would appear to suggest that those antibodies that bind in a complex independent manner would have a higher chance of yielding pathological damage.

Further work has suggested that the fine specificity of anti-GM1 antibodies has importance as a significant prognostic marker in GBS (Lardone et al. 2010). Here the investigators graded 34 GBS patients into different groups based on a clinical score into mild, moderate and severe clinical phenotypes. No difference in ELISA titre was noted between in the groups but in contrast those patients with monospecific IgG (i.e. recognizing GM1 alone as opposed being “cross-reactive” or “polyreactive” recognizing GM1 and GD1b) were more likely to have a severe clinical phenotype.

In our study 13 patients demonstrated IgG which bound GM1 on the combinatorial array. 7 of these were cross reactive with GD1b. 5 of the remaining 6 bound GM1 and no other single glycolipid. The remaining serum demonstrated reactivity to GM1 and GT1a (see Figure 3.4. (A)). As we now know that GT1a was contaminated with GM1 it is likely that this remaining sample was actually monospecific for GM1. In contrast to Lardone's work there was no significant difference in severity (MRC scores, need for ventilation) between these two groups of patients.

To date most studies investigating anti-glycolipid antibody responses have involved conventional assays of single glycolipid antigens using ELISA or TLC. The observation that IgG could preferentially bind to pairs or complexes of glycolipids in GBS was first described 6 years ago in Japanese patients (Kaida et al. 2004). Further work by the group suggested that those patients with anti-glycolipid complex antibodies were clinically distinct with these patients having antecedent gastrointestinal infection and lower cranial nerve deficits more frequently. The presence of IgG to complexes created by GD1b with either GD1a or GT1b was significantly associated with severe disability and a need for mechanical ventilation. In our series of 180 these particular glycolipid complexes did not appear to be significant target antigens with only 1/180 GBS patient demonstrating IgG to the complexes created by GD1b:GD1a and GD1b:GT1b.

Use of the combinatorial array has allowed us to observe different patterns of antibody binding with 26/42 seropositive patients binding preferentially to pairs or complexes of lipids including combinations of sulfatide with GM1/GD1b/GT1a and LM1/GM1 with 15 of these sera demonstrating only reactivity to complexes alone (see Table 3.7. for specificity of these anti-ganglioside complex antibodies). The 26 ganglioside complex seropositive patients represented 8 patients with AIDP, 1 with AMAN and 13 with

equivocal findings. Neurophysiological data was not available the remaining 4 patients. The rest of the seropositive patients included 2 patients with AIDP, 2 with AMAN and 9 with equivocal findings. Data was unavailable for 3 patients.

Whilst the glycolipid positive cohort had significantly lower MRC scores at presentation (see Figure 3.13.) this effect was diminished by week three. Furthermore those patients with anti-complex antibodies were no different to the rest of the seropositive cohort. This is in contrast to earlier reports of anti-complex antibodies aiding the stratification of patients clinically(Kaida et al. 2004)(Kaida et al. 2007).

Sulfatide was noted to be a frequent partner in glycolipid target complexes (see Table 3.7). Sulfatide is a major glycosphingolipid of the myelin sheath in both central and peripheral nervous systems. Synthesized by the Schwann cell in the periphery, sulfatide is a very simple small molecule compared to other glycolipids such as gangliosides. It is composed of galactocerebroside (consisting of a galactose molecule and a ceramide) in which the 3'OH moiety on galactose is sulfated(Norton 1977). Sulfatide deficient and abundant animal models have been created to further investigate the role of this lipid. Roles suggested for this lipid include the maintenance of myelin and node of Ranvier structure amongst others(Eckhardt 2008). Clearly on the PVDF based array sulfatide interacts with glycolipids to reveal targets of interest for IgG. Whether this is a phenomenon that is specific to the array is as yet unknown. Further studies to investigate this should include parallel ELISA studies to investigate whether this finding is simply a reflection of the platform upon which the lipids are presented.

From my data it can be observed that there is variation in the findings between the two methods of ELISA and combinatorial array. This variation in specificity between ELISA and the combinatorial blot is almost certainly a reflection of the platform upon which the lipids are presented. Compared to a plastic ELISA plate, PVDF is a porous substance and one may hypothesize that when lipids are presented using this platform they create conformations that are different to those created on an ELISA plate. Which technique more closely relates that of human myelin is as yet unclear. Further work comparing the binding specificities of a large cohort of seropositive patients may help define these differences further.

This study has several drawbacks including the limited number of controls and the high number of patients within the GBS group with equivocal results on neurophysiological testing. However the data demonstrates that this method is capable of detecting glycolipid antibodies in sera and it is now possible to screen a large number of potential antigens in their combinatorial complexity to help identify the target antigens in GBS.



## Chapter 4. Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)

### 4.1. Introduction

As outlined in Chapter 1, section 1.4., CIDP is considered to be an inflammatory neuropathy with the exact pathogenesis remaining unclear. A small proportion of CIDP patients are found to have anti-ganglioside antibodies (Hughes et al. 2006). A number of immunomodulatory therapies are effective in CIDP including plasmapheresis giving some circumstantial evidence that an auto-antibody mediated mechanism is involved in the pathology of this disease (Dyck, Daube, O'Brien, Pineda, Low, Windebank & Swanson 1986a) (Dyck et al. 1994). Combinatorial lipid arrays have shown that epitope recognition by such antibodies can be dependent on *cis* interaction with other lipids that form heterogeneous microdomains in plasma membranes (Rinaldi et al. 2009). I have used this approach to investigate anti-glycolipid antibody specificities in sera from CIDP patients.

### 4.2. Methods

#### 4.2.1. ELISA

Sera were assayed by ganglioside ELISA as described in section 2.2.3. A positive binding result was considered to give an OD<sub>490nm</sub> of 0.1 or above. All samples were assayed investigating IgG and IgM reactivities to GM1, GM2, GD1a, GD1b and GQ1b. If a positive binding result for any of these gangliosides was observed then the sample was assayed on a wider plate of glycolipids to achieve a final titration. This more complete screen included the gangliosides first assayed (GM1, GM2, GD1a, GD1b, GQ1b) and GD3,

GM3, and GA1. Details of where lipids were purchased are outlined in Chapter 2, Table 2.1.

#### 4.2.2. MAG ELISA

Anti MAG assay is performed with Bühlmann anti-myelin associated glycoprotein IgM ELISA kit. Reagents supplied with this kit include microtitre ELISA plate precoated with human MAG, plate sealer, wash buffer concentrate, incubation buffer, calibrators A-D, low and high control, enzyme labelled IgM (anti-human IgM antibody conjugated to HRP), TMB substrate (TMB in citrate buffer with hydrogen peroxide) and a stop solution (0.02M sulphuric acid). Serum samples are diluted to 1:1000 in incubation buffer (2µl sample + 2µl buffer). Samples are vortexed then incubated for 1 hour at room temperature. Samples are then placed on ice for 10 minutes. Test strips (wells) are placed onto the plate holder and washed 4x with wash buffer. 100 µl of incubation buffer, samples, calibrators (A-D) and high and low controls are added to each well. The plate is then covered with the plate sealer and incubated at 2-8°C. After 2 hours empty wells and wash 4x with wash buffer and pat dry. Then add 100 µl per well of HRP-labelled IgM. Cover with plate sealer and incubate at 2-8°C. After 2 hours empty wells and wash 4x with wash buffer and pat dry.

Allow substrate to reach room temperature and then add 100 µl per well and cover with plate sealer. Incubate in darkness on a rocking shaker at room temperature. After 30 minutes add 100 µl per well of stop solution. Read on plate reader, Dynatech Laboratories MR500, programme 7 (dual wavelength of 450nm test filter/630nm reference of background filter). A standard curve is constructed from the standards blank and A-D. From this graph it is verified that the high and low controls fall within specified range. Results are reported as positive if >1000 BTU (Bühlmann Titre Units) and negative if < 1000 BTU. Anti- MAG ELISA experiments were carried out by Ms

Emma Lewis, Neuroimmunology laboratory, Southern General Hospital, Glasgow.

#### 4.2.3. Combinatorial array

Using the Camag Automatic TLC Sampler 4, I assembled an array of individual glycolipids and their 1:1 combinations (complexes) spotted onto polyvinyl-difluoride (PVDF) membranes affixed to microscope slides. I investigated the serum IgG and IgM response to 9 individual lipids including sulfatide (Sulf), galactocerebroside (GalC), GM1, GM2, GD3, GD1a, GD1b, GT1b, GQ1b and all their possible 1:1 complexes thus comprising 45 target antigens in total. Details of where lipids were purchased are outlined in Chapter 2, Table 2.1. We probed these prepared slides with 57 CIDP serum samples, 30 serum samples from patients with Multiple Sclerosis and 27 serum samples from healthy controls. Each serum was assayed at a concentration of 1:100 unless otherwise stated. Antibody binding to specific lipids was then detected using standard chemiluminescence and autoradiography using the protocol as outlined in Chapter 2; 2.2.4.

#### 4.2.4. Patients

Serum IgG and IgM lipid reactivities were investigated in CIDP patients (n=57), Multiple Sclerosis patients (MS) (n= 30) and healthy controls (HC) (n= 27). Sera were obtained from CIDP patients taking part in the randomized controlled trial of methotrexate for chronic inflammatory demyelinating polyneuropathy(RMC Trial 2009). The 57 CIDP patients were enrolled from 26 European centres and were eligible for entry if they were at least 18 years of age, had been diagnosed as having CIDP by a consultant neurologist,

and had a chronically progressive, stepwise or recurrent weakness of all extremities, with absent or reduced tendon reflexes and with or without sensory dysfunction developing over at least 2 months and present for at least 6 months. Patients had to meet the diagnostic criteria for definite or probable CIDP within the previous 3 years (Hughes R. Bensa S. Willison H. Van den Bergh P. Comi G. Illa I. Nobile-Orazio E. van Doorn P. Dalakas M. Bojar M. Swan A. Inflammatory Neuropathy Cause and Treatment (INCAT) Group 2001) (Joint Task Force of the EFNS and the P.N.S. 2005). All patients were receiving a stable dose of immunoglobulin or corticosteroids or both. CIDP patients were randomly allocated treatment with weekly methotrexate in a double blinded fashion (32 placebo; 25 methotrexate). Exclusion criteria included the presence of IgM paraprotein with antibodies to myelin associated glycoprotein (MAG) however patients with other paraproteins were not excluded. Serum samples were donated at enrolment and at end of trial. Certain clinical information and neurophysiological data (e.g. CMAP, distal motor latency, conduction velocities) was available in an excel database.

All MS (30) patients met the McDonald diagnostic criteria for MS (Polman et al. 2005) and included 24 relapsing remitting MS (RRMS), 5 primary progressive MS (PPMS) and 1 secondary progressive MS (SPMS). All MS patients were immunomodulatory treatment naïve. The 27 healthy controls were volunteers without symptoms or history of neurological disease. CIDP patients were older compared to the other two groups and had a higher proportion of men (Table 4.1). All sera were separated from clotted blood and stored at -70°C until use.

Table 4.1. Characteristics of CIDP patients

	CIDP	MS	HC
<i>N</i>	57	30	27
Sex			
Male	40*	13	7
Female	17	17	20
Age (y)			
Median	58†	38.5	35
Range	47-64	29.5-45.4	30-47

Age and sex for the patient subgroups. Data are presented as median (IQR-interquartile range).

\*  $p < 0.05$  and  $p < 0.001$  if compared with MS or HC respectively ( $\chi^2$  test)

†  $p < 0.0001$  if compared with MS or HC (ANOVA with Bonferroni correction for multiple comparisons)

#### 4.2.5 Statistical analysis

ANOVA analyses were used to compare anti-glycolipid levels between more than three groups. The Mann-Whitney test was used to compare anti-glycolipid antibody levels between two groups. Paired t tests were used to detect if anti-complex antibodies were of higher intensity than antibodies to the contributing single lipid. Differences in proportions were tested for significance by chi-squared test and Fisher's exact test where appropriate. These analyses were performed with PRISM4 (GraphPad software, USA). Raw data was logarithmically transformed (log 2) and heatmaps, hierarchical clustering and further ANOVA analyses were performed by Dr Gabriela Kalna of the University of Glasgow using Partek Geometrics Suite (Partek Incorporated, USA).

### 4.3. Results

#### 4.3.1. ELISA

Using the ELISA assay anti-glycolipid antibodies (AGAs) were not demonstrated significantly more frequently in the CIDP group. The data shows that 13/57 (22.8%), 2/30 (6.7%) and 3/27 (11.1%) serum samples from CIDP, MS and HCs respectively have elevated levels of IgM (AGA) anti-ganglioside antibodies (Figure 4.1 (A)). This compares to IgG responses detected in 2/57 (3.5%) CIDP, 0/30 MS (0%) and 2/27 (3.5%) HC.

However the number of CIDP IgM AGAs is significantly higher than the number of CIDP IgG AGAs ( $p=0.0042$ , Fisher's exact test). Indeed those sera which were positive for IgG AGA's had only borderline positive ODs with much higher IgM OD's observed for the same ganglioside. Presumably the positive OD's seen in this CIDP group reflect the polyvalent binding capabilities of the IgM AGAs.

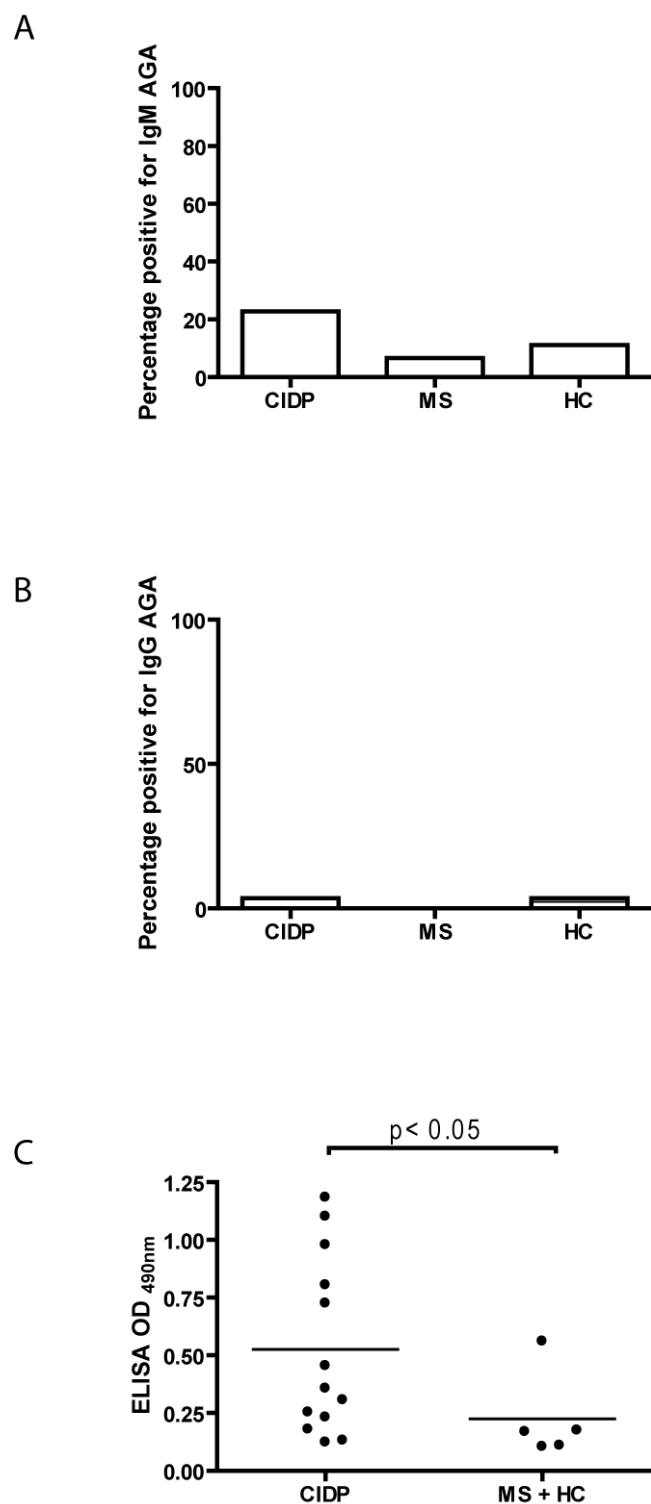
The number of IgM AGA positive sera was not significantly higher ( $\chi^2$ ) compared to the MS and HC groups. However within those patients who had ODs>0.1, the actual OD's observed for the CIDP group were higher compared to those observed in the combined MS and HC group (Figure 4.1,  $p=0.0487$ , Mann Whitney). The presence of IgM anti-ganglioside antibodies in our healthy controls may reflect the presence of laboratory workers with exposure to gangliosides in our healthy control population. Indeed exogenous gangliosides used to treat neurological diseases have been associated with the both the development of IgG against gangliosides and the development of GBS(Landi et al. 1993).

IgM directed against GD1b was the only antibody seen specifically more frequently in the CIDP group compared to the combined groups of MS and HCs (see Figure 4.2 and Figure 4.3) ( $p < 0.05$ , ANOVA with Bonferroni correction for multiple comparisons). These GD1b reactive sera were clinically and neurophysiologically indistinct from the rest of the CIDP cohort. Table 4.2 lists the final titres obtained for IgM AGAs in the CIDP cohort.

Only 3/13 samples demonstrated binding to one glycolipid only via ELISA. This specific binding was not restricted to one lipid but to two individual glycolipids- GM1 (Serum 18 & 91) and GD1b (Serum 28). The rest of the AGA positive sera bound to a variety of glycolipid antigens (for details see Table 4.2.).

Antibody positive patients did not differ clinically from antibody negative patients. Table 4.3 lists the clinical details of the individual AGA positive patients. Table 4.4. lists the characteristics of antibody positive and negative patients. Furthermore neurophysiological measurements including CMAP amplitude, motor conduction velocities and distal latencies were similar across the two groups (see Figure 4.4, 4.5 & 4.6.). This similarity between the two groups may be a result of our small sample size but this may reflect that AGA positive patients have a similar clinical picture to AGA negative patients. In addition pathologically there is no evidence that the AGA positive patients are differ from AGA negative patients (Toyka et al. 2003) (Hughes et al. 2006). One may make the assumption therefore that we are not looking for the correct antigen(s). Increasing the number of antigens and also investigating responses against complexes of glycolipids by employing the technique of combinatorial array may help therefore to identify additional antibody positive patients.

One sample (36) bound MAG via ELISA with a titre of 7400 BTU. Interestingly this sera bound GM2 and GA1 with titres of 5000 and 10000 respectively.



**Figure 4.1. ELISA binding ratios for AGAs and ODs of AGAs observed.**

(A) Percentage of sera positive for IgM AGA's in CIDP, MS and HC groups.

(B) Percentage of sera positive for IgG AGA's in CIDP, MS and HC groups.

(C) OD's of the detected AGA's in CIDP and combined MS and HC groups. In the samples where there is more than one AGA the highest OD value is used.



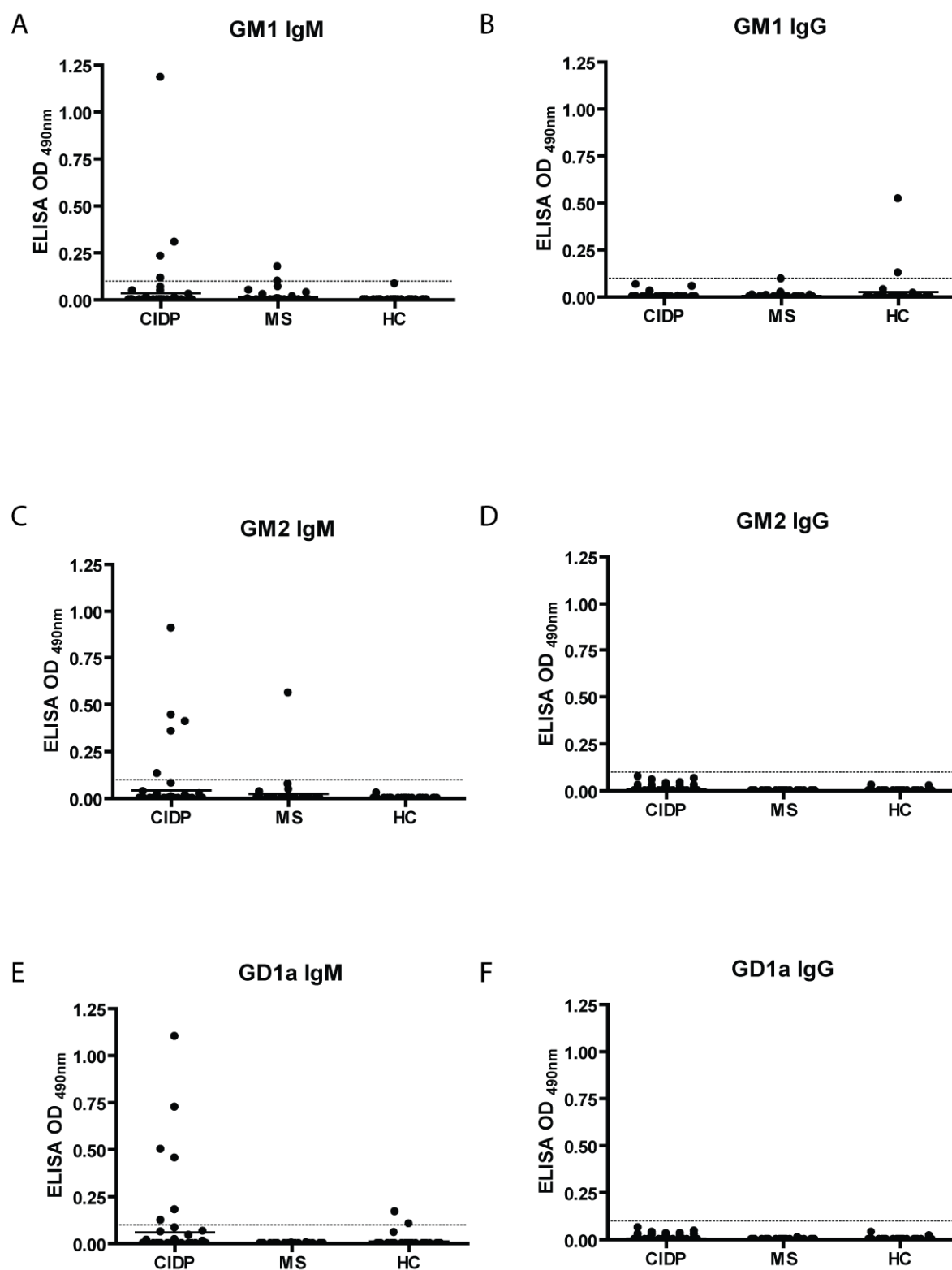


Figure 4.2 AGA frequencies for GM1, GM2 and GD1a

(A)- (F) IgM and IgG AGA frequencies for GM1, GM2 and GD1a across all groups. The dotted line intersects the Y axis at 0.1.

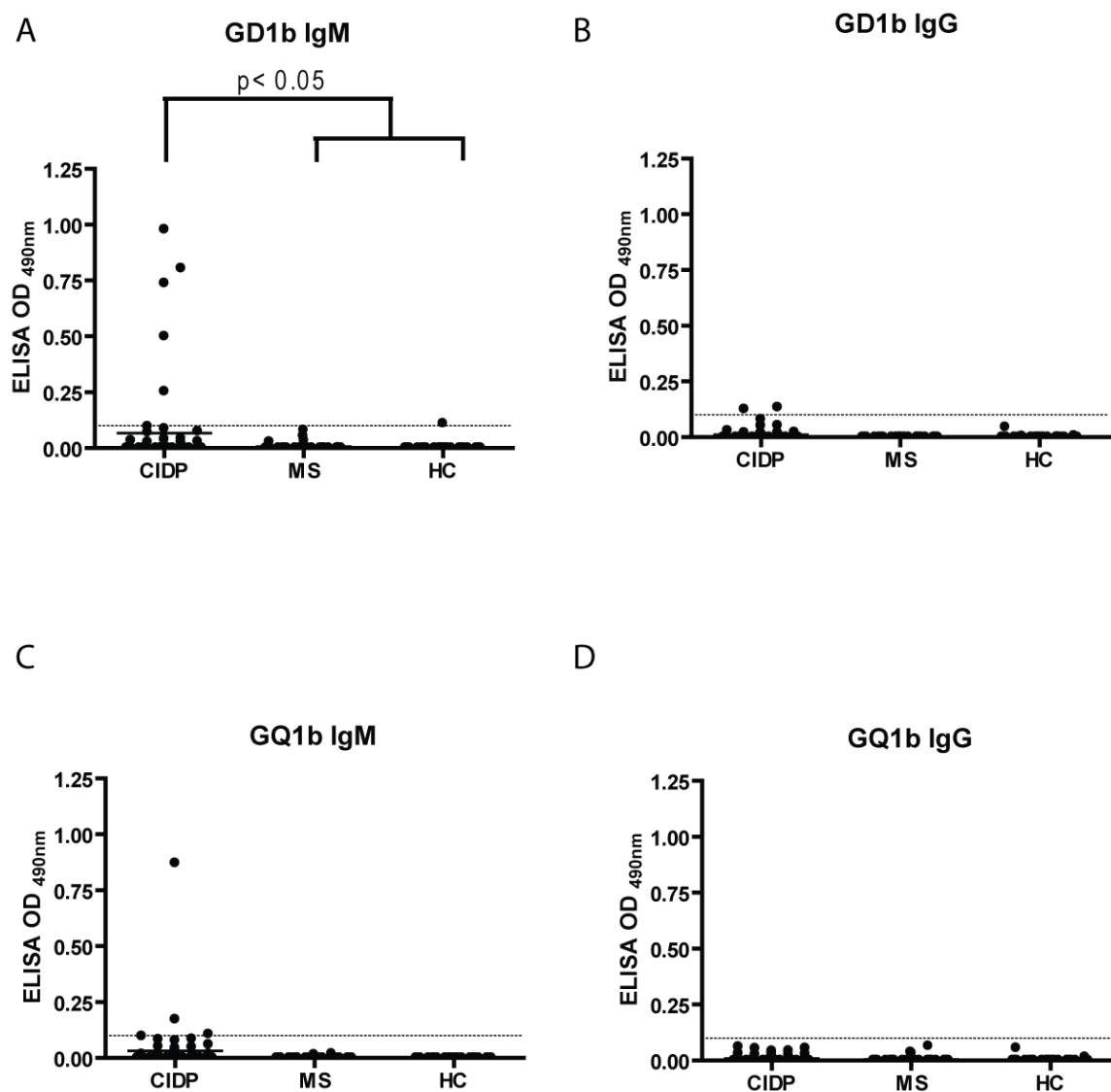


Figure 4.3 AGA frequencies for GD1b and GQ1b.

(A)- (D) IgM and IgG frequencies for GD1b and G1b across all groups. The dotted line intersects the Y axis at 0.1. IgM antibodies to GD1b are found more frequently in the CIDP group compared to combined group of MS and HC ( $p < 0.05$ , ANOVA with Bonferroni correction for multiple comparisons)

Table 4.2. IgM anti-ganglioside reactivities of individual CIDP patients

ID	GM1	GM2	GAl	GD3	GM3	GD1a	GD1b	GT1b	GQ1b
13	12500	2000	12500	0	0	0	12500	0	300
18	500	0	0	0	0	0	0	0	0
26	0	0	0	0	12500	12500	0	12500	0
28	0	0	0	0	0	0	12500	0	0
31	1000	12500	0	1500	12500	800	10000	12000	600
35	160	1000	2500	300	1400	0	160	700	0
36	0	5000	10000	0	0	0	0	0	0
63	0	0	200	0	2700	2500	0	2200	0
67	0	2500	150	0	0	4600	250	2500	0
73	0	0	150	900	0	200	200	200	0
75	1100	180	2400	0	140	0	12500	0	0
82	0	0	0	12500	700	12500	12500	12500	12500
91	4000	0		0		0	0	0	0

Table 4.3. Clinical details and anti-ganglioside specificity for IgM seropositive CIDP patients.

ID	Sex	Age	Disease duration in years	ONLS	*AGA specificity
13	M	41	1	4	GD1a, GA1, GD1b
18	M	64	19	2	GM1
26	M	64	11	4	GM3, GD1a, GT1b
28	M	61	4	5	GD1b
31	M	49	29	5	GM2, GM3
35	M	43	0.75	3	GA1
36	M	65	6	3	GA1
63	F	67	3	2	GM3
67	M	38	14	4	GD1a
73	M	80	5	6	GD3
75	M	44	9	3	GD1b
82	F	69	20	8	GD3, GD1a, GD1b, GT1b, GQ1b
91	M	47	6	5	GM1

\* IgM AGA with highest titre. For full details of specificity see Table 4.2.

M=male; F=female; Duration is presented in years; ONLS= overall neuropathy limitations scale (higher values indicate higher disability- range 0-12); AGA= anti-glycolipid antibody;

Table 4.4. Characteristics of anti-glycolipid antibody (AGA) positive and negative CIDP patients.

	AGA positive (n=13)	AGA negative ( n=44)	Total (n=57)
Men	11 (85%)	29 (66%)	40 (70%)
Age (years)	61 (43.5- 66)	58 (47.5-63.5)	58 (47-64)
Duration of disease (years)	7.5 (4.5-19.5)	7 (4-13.5)	7 (4-15)
MRC sum score ( range 0-80)	65 (55-76.5)	72 (67.5-80)	72 (65-76)
ONLS score (range 0-12)	4 (3-5.5)	4 (2.5-4.5)	4 (3-5)
Upper limb ONLS score (range 0-5)	3 (2-3)	2 (1-2)	2 (1.5-3)
Lower limb ONLS score (range 0-7)	2 (1-2.5)	2 (1-2)	2 (1.5-3)
Sensory sum score (range 0-64)	7 (0-19)	9.5 (4-20)	9 (4-20)

Data are number (%) or median (IQR). MRC sum score (possible range 0-80) higher values indicate greater strength; Sensory sum score (possible range 0-64) higher values indicate greater impairment. There was no statistical difference in clinical phenotype between the antibody positive and negative groups (Mann-Whitney).

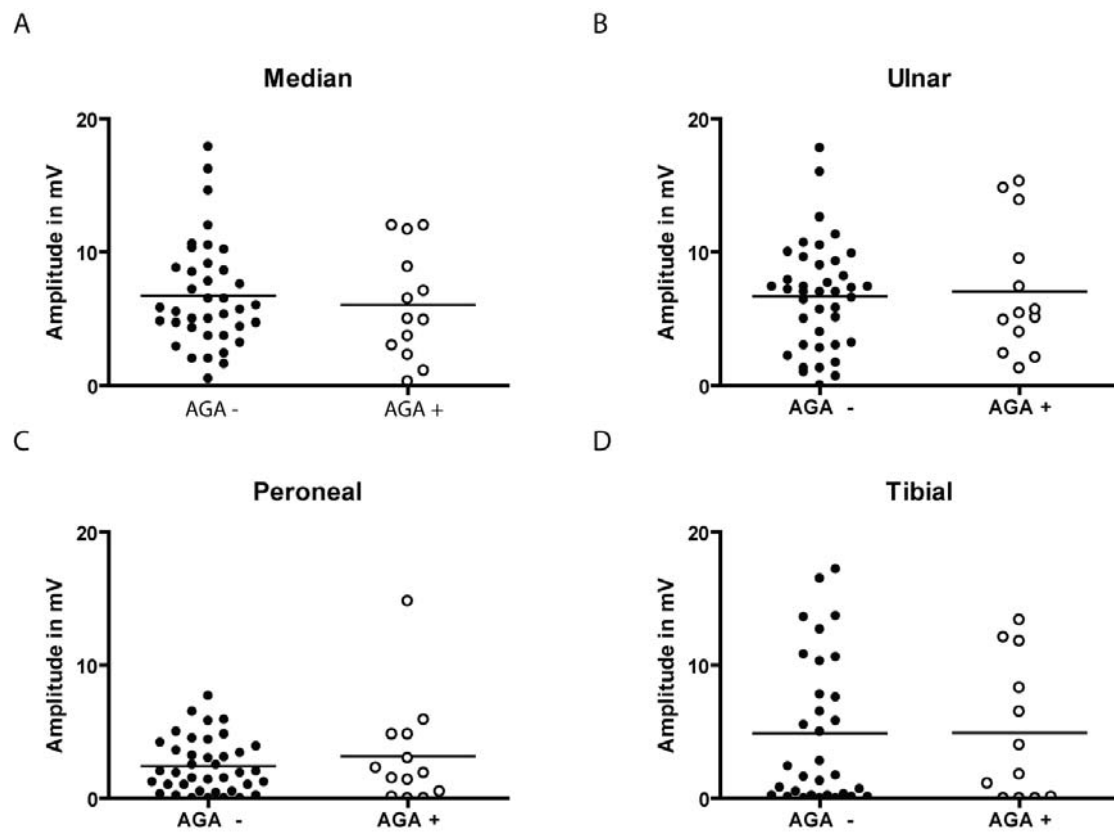


Figure 4.4. CMAPs in antibody negative (AGA -) and antibody positive (AGA +) patients.

One CMAP measurement per patient.

- A. Median nerve
- B. Ulnar nerve
- C. Common peroneal nerve
- D. Tibial nerve

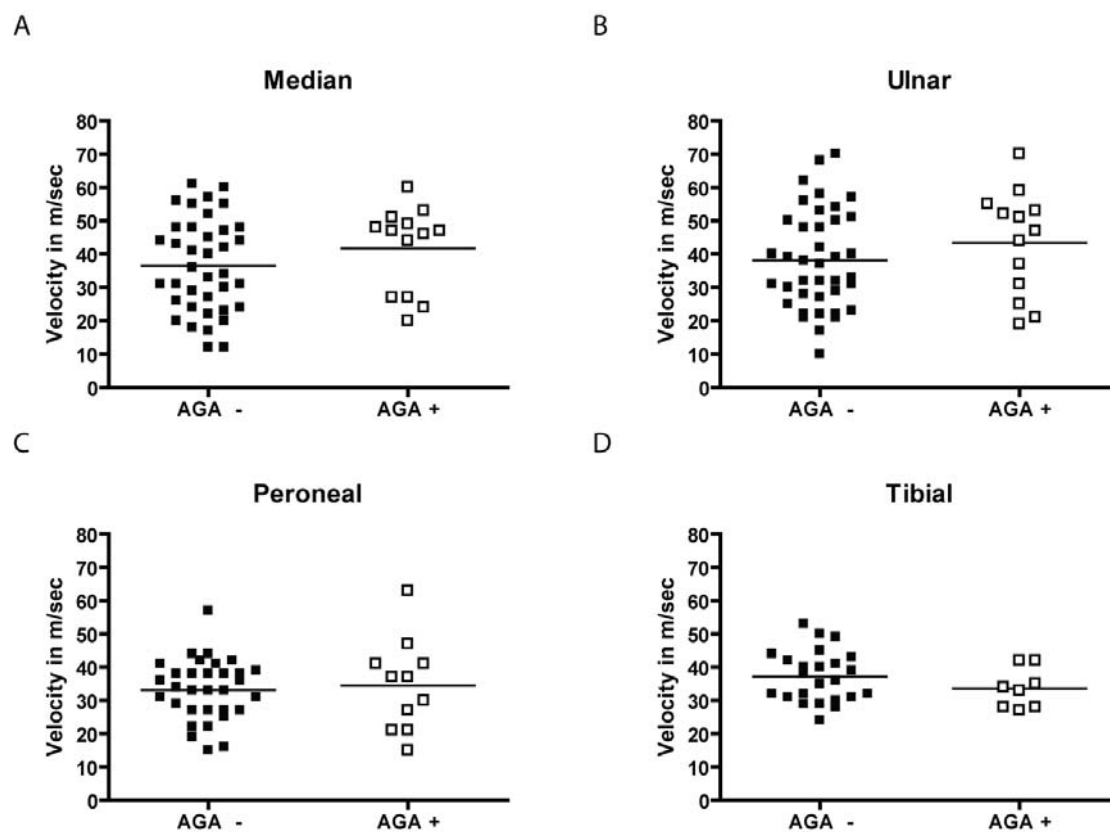


Figure 4.5. Motor conduction velocities in antibody negative (AGA-) and antibody positive (AGA+) CIDP patients

- A. Median nerve
- B. Ulnar nerve
- C. Common peroneal nerve
- D. Tibial nerve

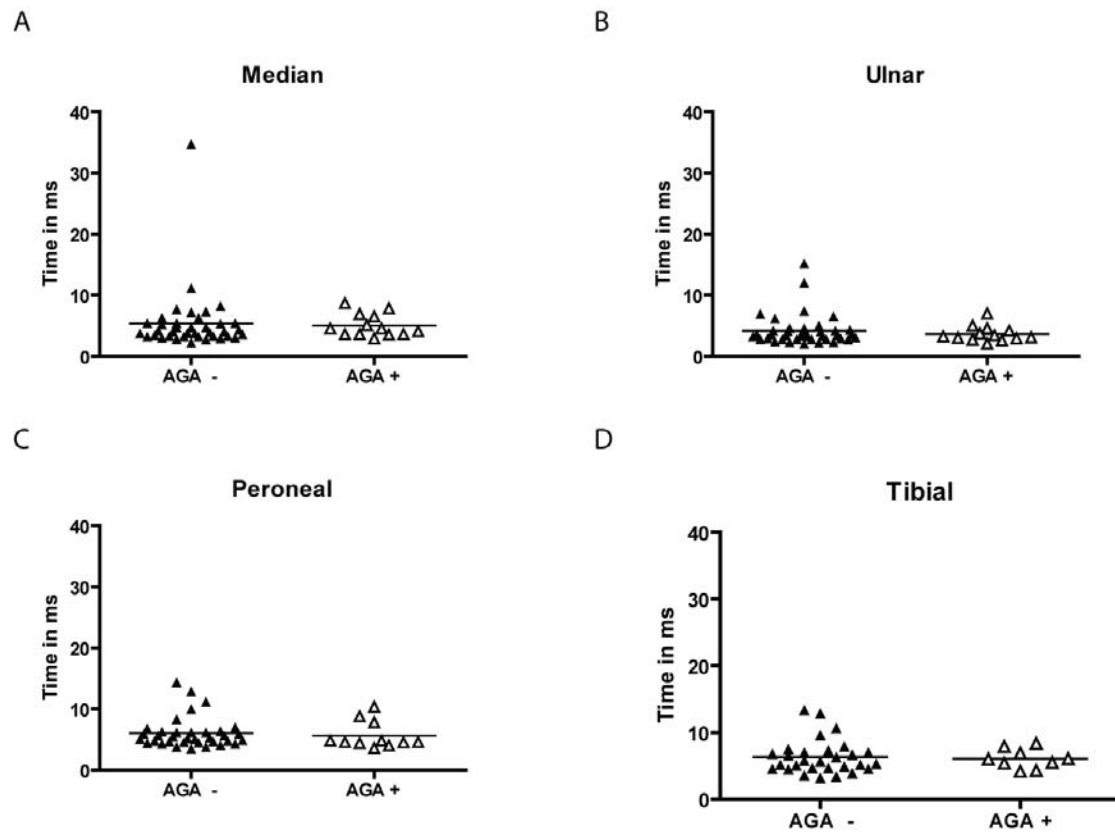


Figure 4.6 Distal latencies in antibody negative (AGA-) and antibody positive (AGA+) CIDP patients

- A. Median nerve
- B. Ulnar nerve
- C. Common peroneal nerve
- D. Tibial nerve



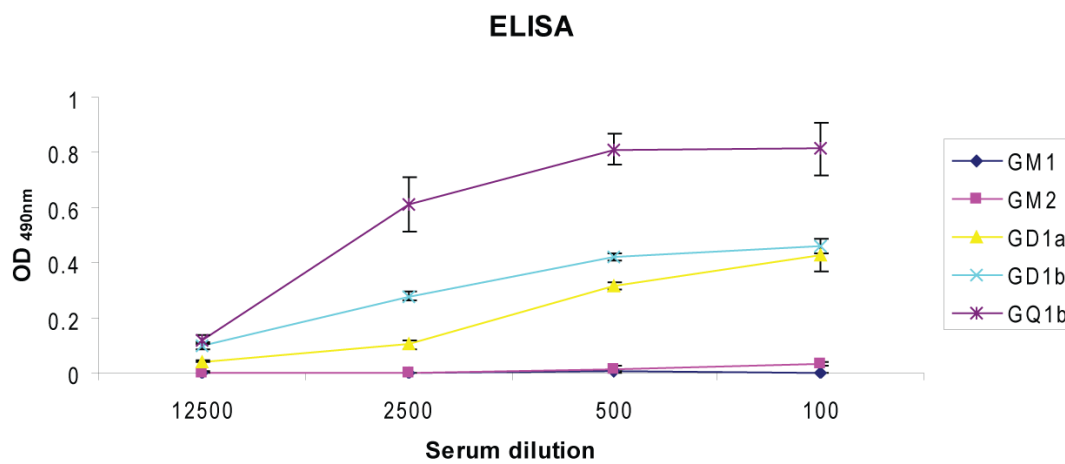
### 4.3.2. ELISA vs. combinatorial array

Initially I compared our combinatorial method to ELISA. Inter- and intra-assay variation did not statistically differ between the two methods (see Methods chapter). The control serum used in all ELISA and array experiments was positive for IgM directed against GD1a, GD1b and GQ1b but negative for antibody directed against GM1 and GM2. Similar results were observed between the two methods (Figure 4.7.). For IgG experiments serum with known IgG reactivities to the gangliosides GM1, GD1b and GT1a demonstrated similar responses using the two methods. These “positive control” sera were used in every “run” of experiments performed.

All samples positive on ELISA for IgM AGA were similarly positive using the combinatorial method. Figure 4.8. displays correlation and Bland-Altman graphs for ELISA and array data observed. Correlation quantifies how well X and Y variables vary together. As the data is not normally distributed Spearman correlation was employed. Two-tail p values were all significant (with the exception of GQ1b) demonstrating a positive correlation between the two methods.

As with the GBS data presented in Chapter 3, I have employed Bland-Altman plots to compare two assay methods (Bland et al. 1986). The graph is constructed so the difference between the two measurements (Y axis) is plotted against the average of the two measurements (X). Once again as our two methods have different units of measurement, optical density and arbitrary units of intensity, the raw data is normalised. The highest value observed in each respective dataset was set as 100% with all other values expressed as a percentage. Whilst this is not ideal this method does give us a better feel of the agreement between the two methods. Correlation between the two methods whilst positive was not particularly strong (see Figure 4.8).

A



B

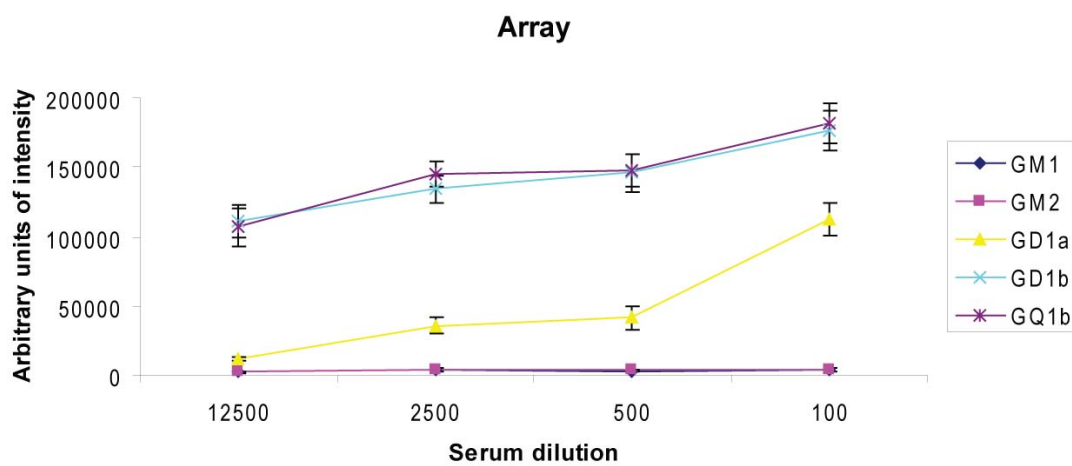


Figure 4.7. ELISA and array dilution of control serum.

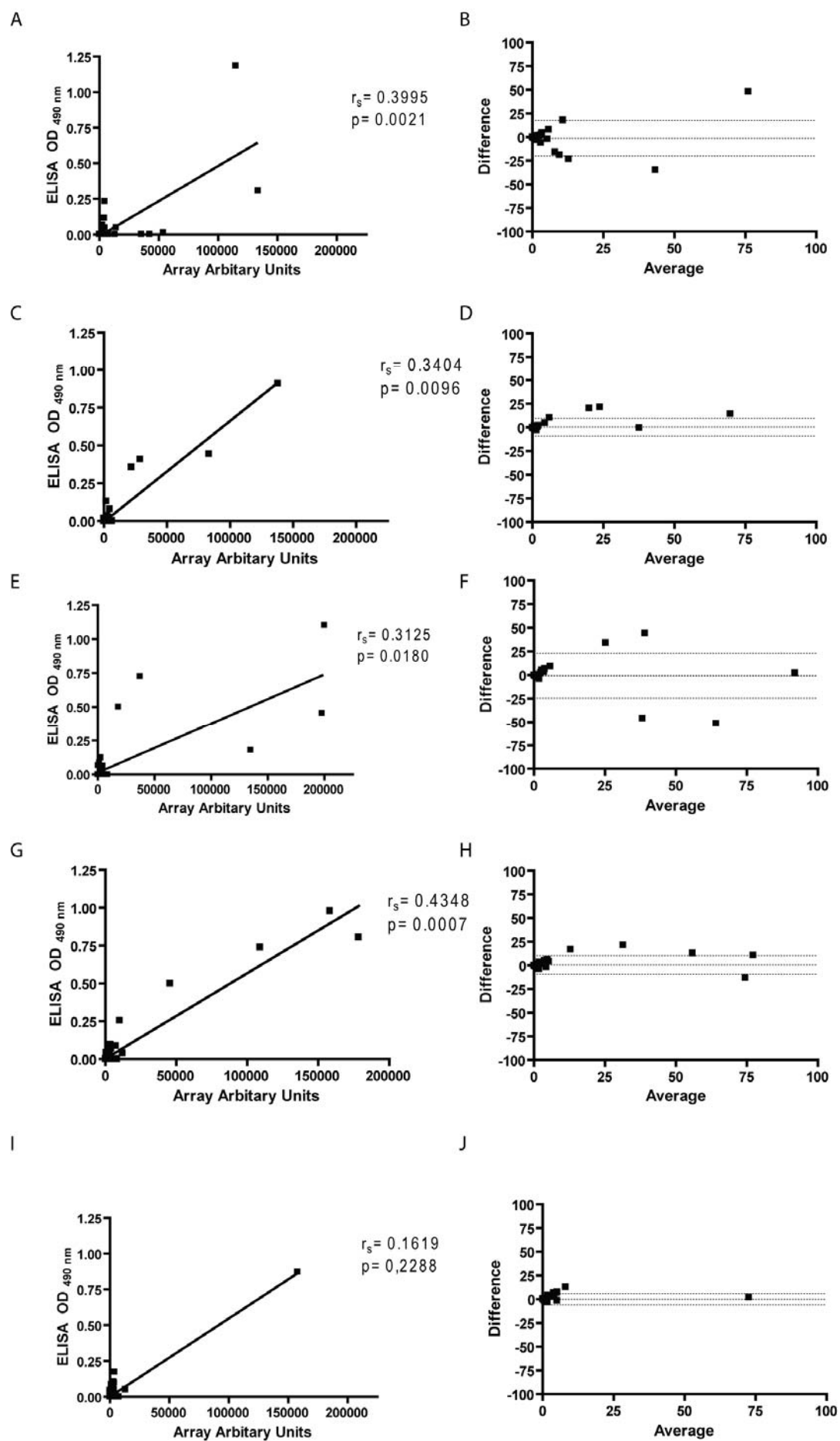


Figure 4.8. Correlation and Bland-Altman graphs.

GM1 (A, B), GM2 (C, D), GD1a (E, F) GD1b (G, H) and GQ1b (I, J).

$r_s$ , the nonparametric Spearman correlation coefficient, has the range -1 to +1. P values quoted are two-tailed p values. The Bland-Altman graphs have three dotted lines intersecting the Y axis, representing the mean difference (middle dotted line) with the dotted lines either side of the mean difference representing the 95% limits of agreement.

### 4.3.3. Combinatorial array

All CIDP samples positive on ELISA were similarly positive using the combinatorial array technique. However the combinatorial array allowed us to identify an extra 6 CIDP patients who demonstrated IgM against a variety of glycolipids (see Table 4.5 and Figure 4.9). IgM reactivity to glycolipids (not including sulfatide) was observed in 19/57 (33.33%) CIDP patients, 4/30 (13.33%) MS patients and 2/27 (7.4%) healthy controls. IgM to glycolipids (not including sulfatide) was seen significantly more in the CIDP group compared to the combined group of MS patients and healthy controls (Fishers exact test,  $p=0.0105$ ).

There was no significant association of IgG or IgM directed against sulfatide or galactocerebroside within the CIDP group using the combinatorial array (see Figure 4.10).

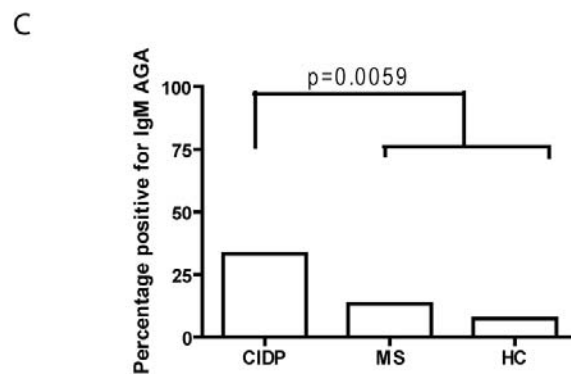
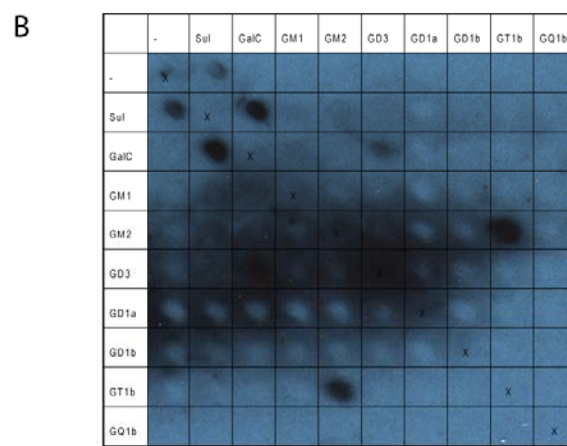
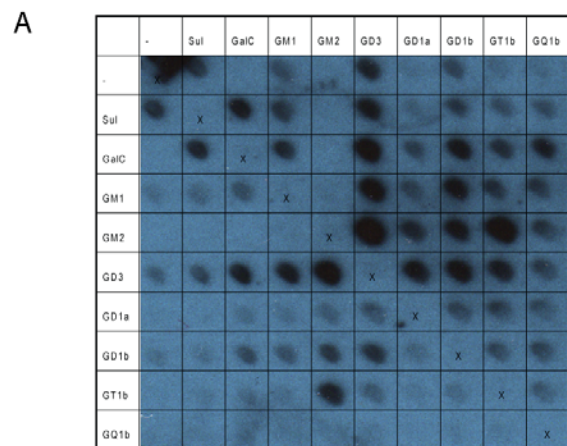
As we are dealing with 45 potential antigens per patient heatmaps have been employed to visualise the raw data. Figure 4.11. and 4.12. illustrate heatmaps obtained using logarithmic transformations of the IgM mean intensities recorded for each lipid antigen. Different measures of hierarchical clustering techniques were applied to the data (with clinical and paraclinical information linked) were applied. Figure 4.11 demonstrates data from IgM reactivities of the CIDP cohort when the Pearson squared clustering technique was employed. Here it can be visualized that the CIDP patients segregate into different groups with a gangliosides reactive group, sulfatide only reactive group and a glycolipid antibody negative group. In keeping with the ELISA data these groups did not differ clinically or electrophysiologically. In contrast to the ELISA data there was not one glycolipid/glycolipid complex antibody demonstrated significantly more frequently in the CIDP cohort. Data from healthy controls and MS patients is presented in Figure 4.13.

Different patterns of antibody binding were observed in the CIDP group. The predominant pattern appeared to be a complex independent pattern of

binding with antibody binding to the glycolipid no matter what glycolipid it was partnered with (Figure 4.14.(A) and Figure 4.15 (A)). In addition attenuation of binding was observed in certain patients (Figure 4.14 (B) and Figure 4.15 (B)). In four patients was enhanced reactivity to complexes observed (Table 4.5.). This was once again defined as reactivity to the complex of lipids being greater than the sum of the reactivities for the two individual complexes and is illustrated in Figure 4.14. (C) (and Figure 4.15 (C)). In all 4 cases binding to single glycolipids was negligible with arbitrary units of detection measuring <10,000.

Table 4.5. Additional sera with detectable IgM glycolipids identified using combinatorial array

Serum ID	IgM glycolipid reactivity
37	GD1b
41	Complexes (GalC:GD3, GM2:GT1b)
43	Complexes (GalC:GD3, GM2:GT1b)
44	GM1
79	Complexes (GD1a:GD1b)
92	GM1



**Figure 4.9** Combinatorial blot; extra samples identified and proportions of patients testing positive for IgM glycolipid antibodies

Row and column headings reveal the complex at each location, “X”s represent the negative controls (methanol) which act as a line of symmetry for duplicate spots within the same membrane.

A. Serum 37 on combinatorial blot

B. Serum 41 on combinatorial blot

C. Percentage of patients demonstrating IgM to glycolipids and their complexes

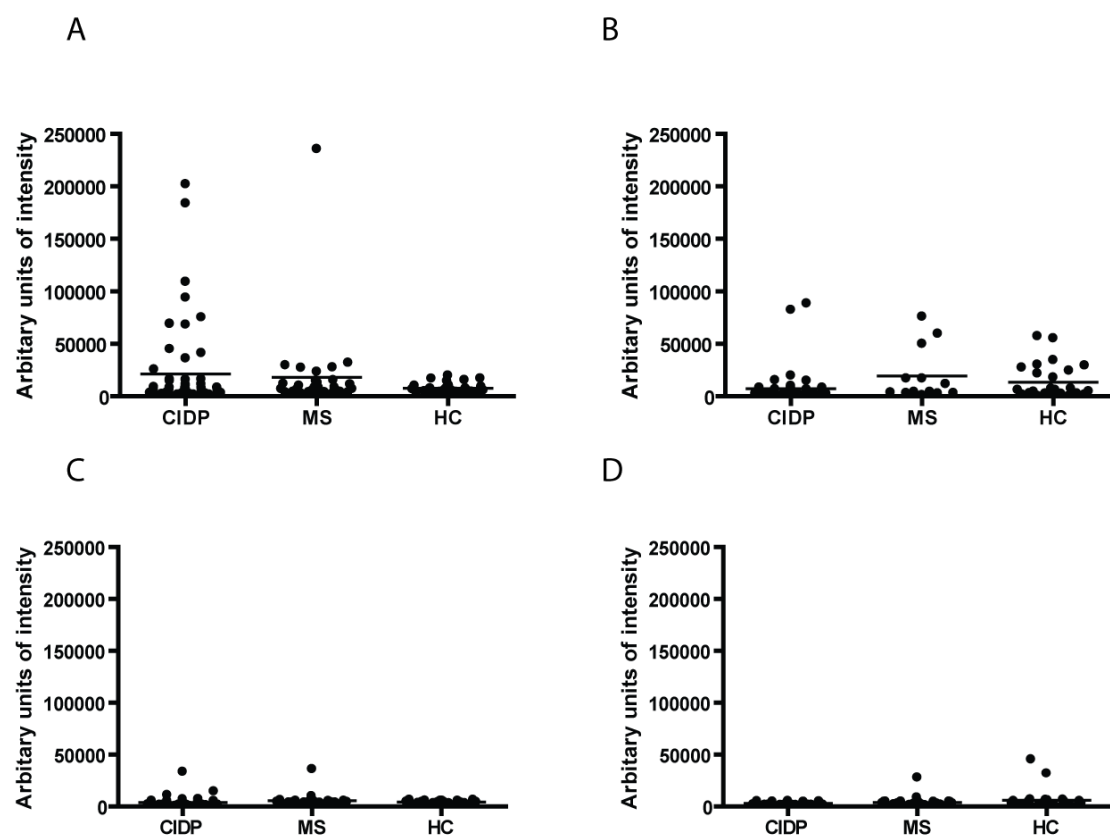


Figure 4.10. IgM and IgG to sulfatide and galactocerebroside.

- A. IgM sulfatide reactivities in CIDP, MS and HC groups.
- B. IgG sulfatide reactivities in CIDP, MS and HC groups.
- C. IgM galactocerebroside reactivities in CIDP, MS and HC groups.
- D. IgG galactocerebroside reactivities in CIDP, MS and HC groups.



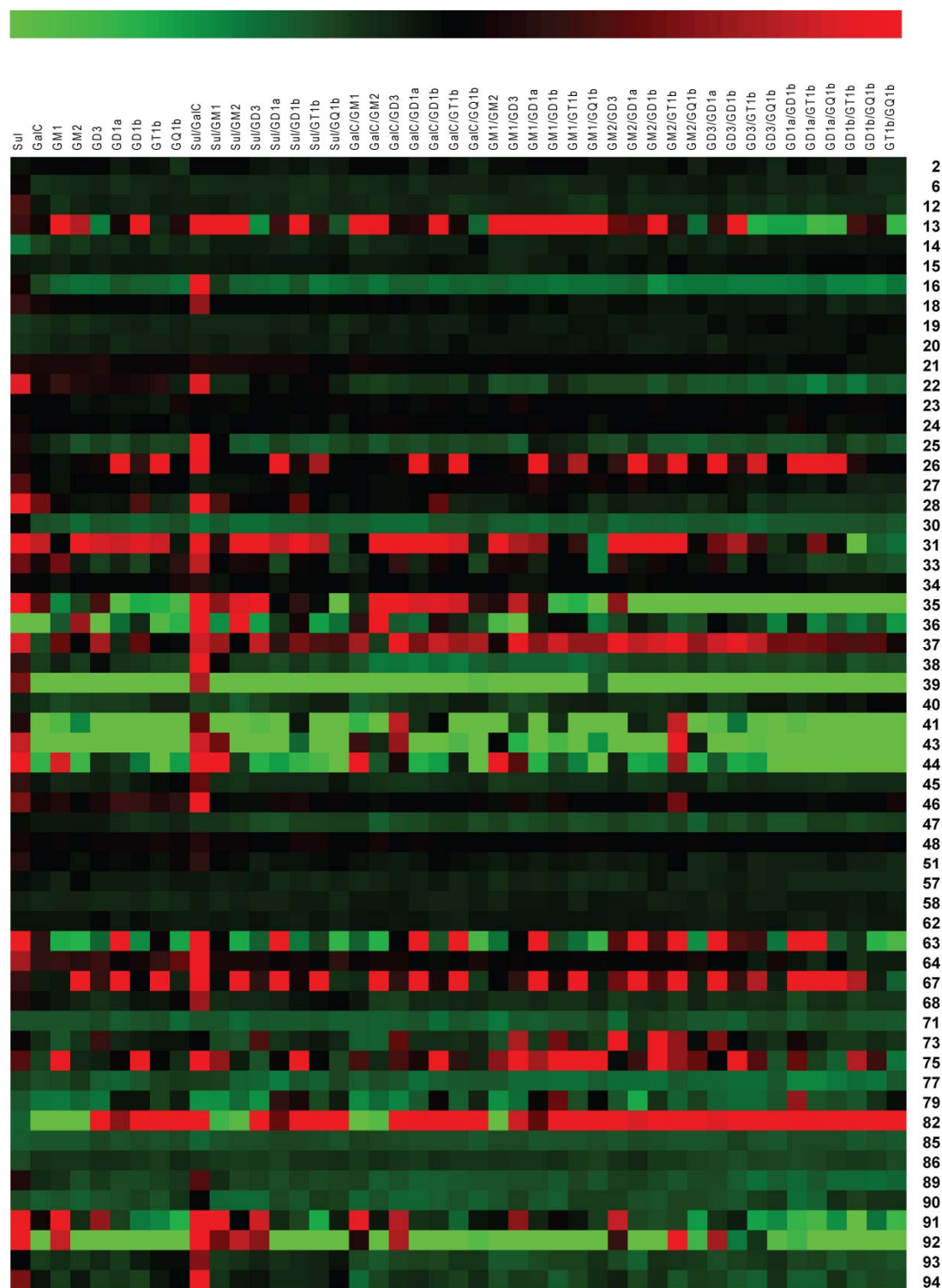


Figure 4.11 IgM Array analysis in CIDP patients.

Heatmaps, created using logarithmic transformations of the mean intensities recorded for each lipid antigen depicting serum IgM reactivity in individual patients. Lipids are displayed as column headings and each row represents an individual patient.

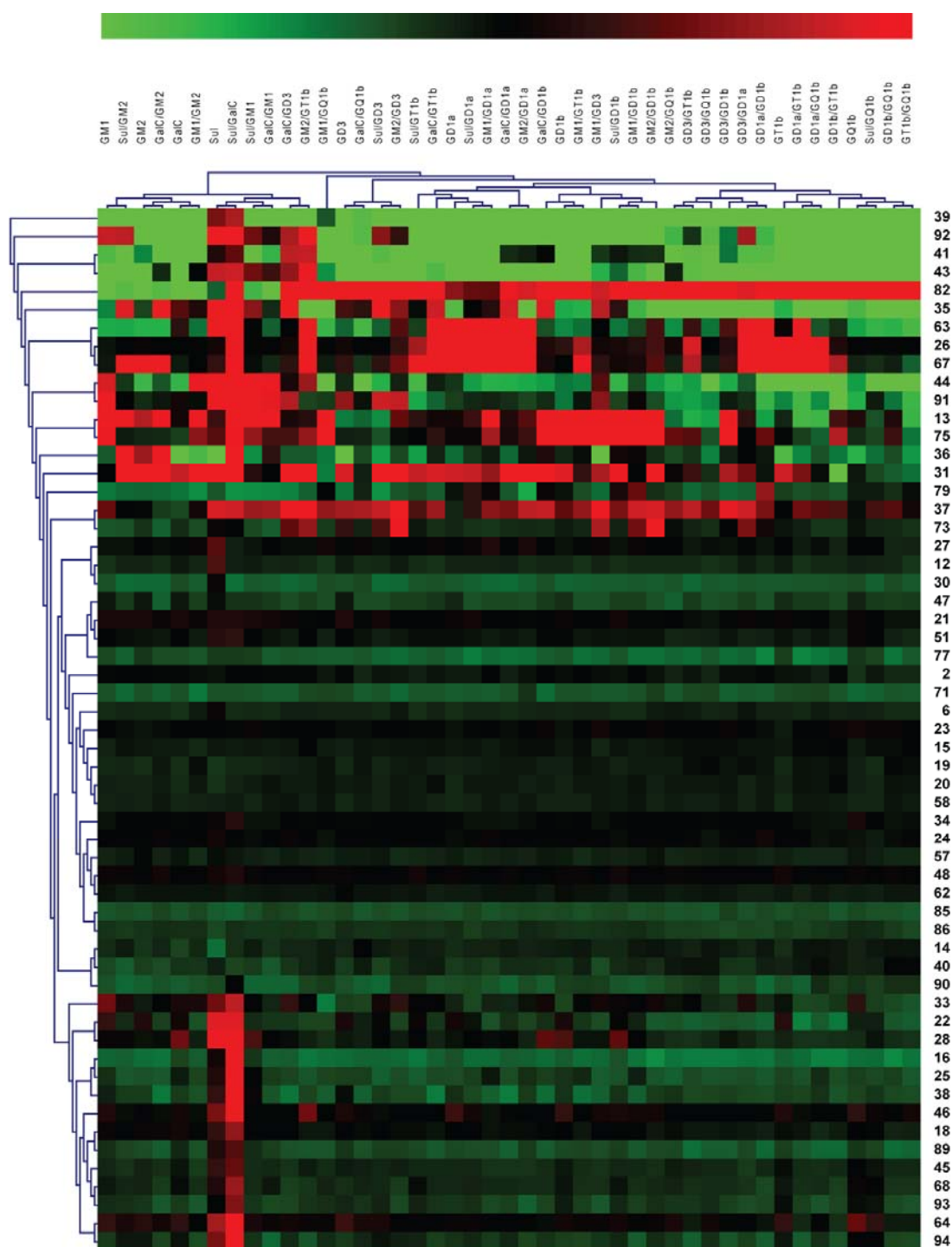


Figure 4.12 Heatmap depicting reactivity of IgM derived from CIDP.

Created as before with logarithmically transformed data, the lipid antigens are displayed as column headings and each row represents an individual patient. Here the data has been clustered using Pearson squared revealing two populations of lipid reactive CIDP IgM, one to gangliosides and one to sulfatide.

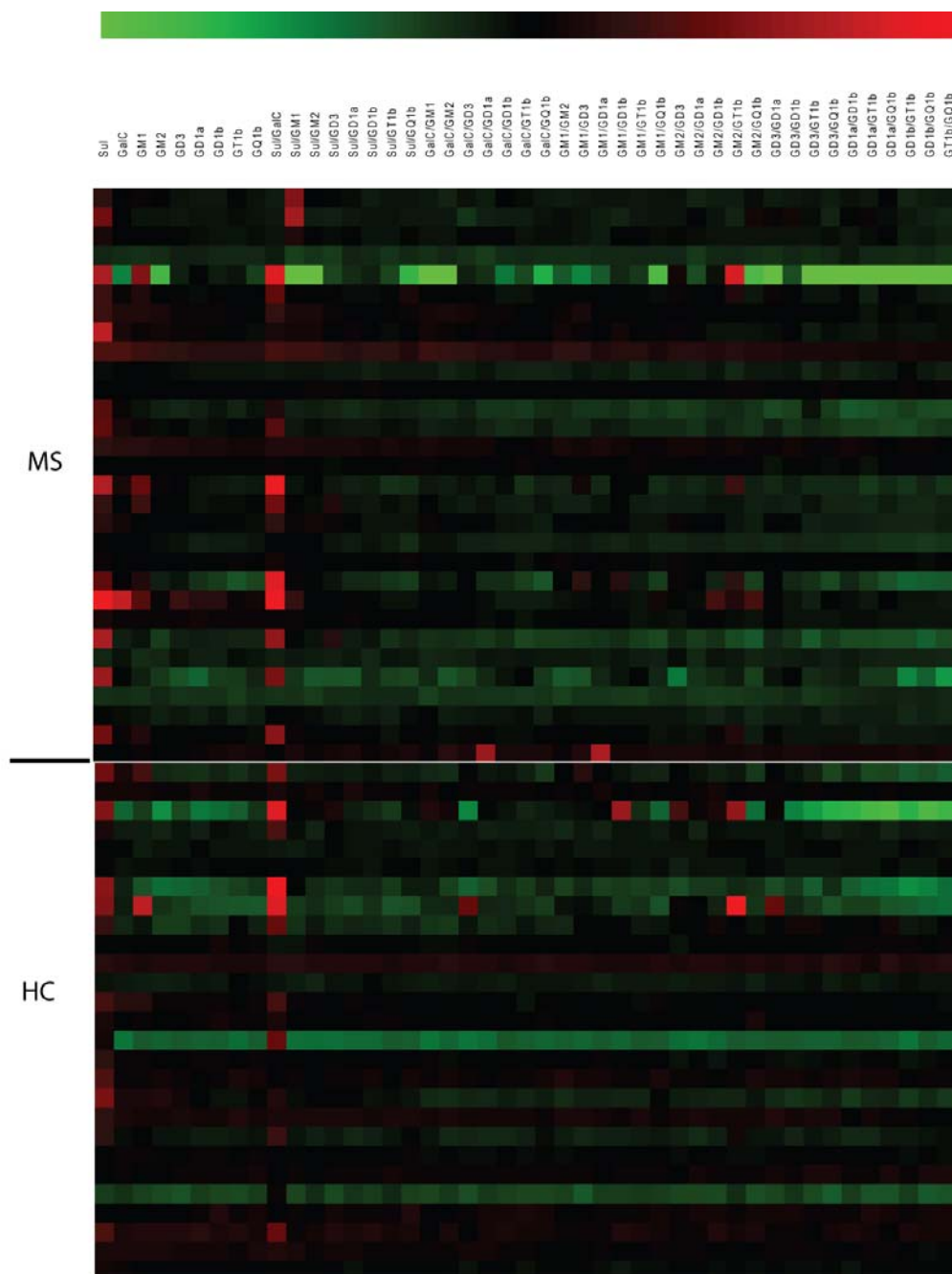


Figure 4.13 Heatmap depicting reactivity of IgM derived from MS and HC. Heatmaps were created as before.

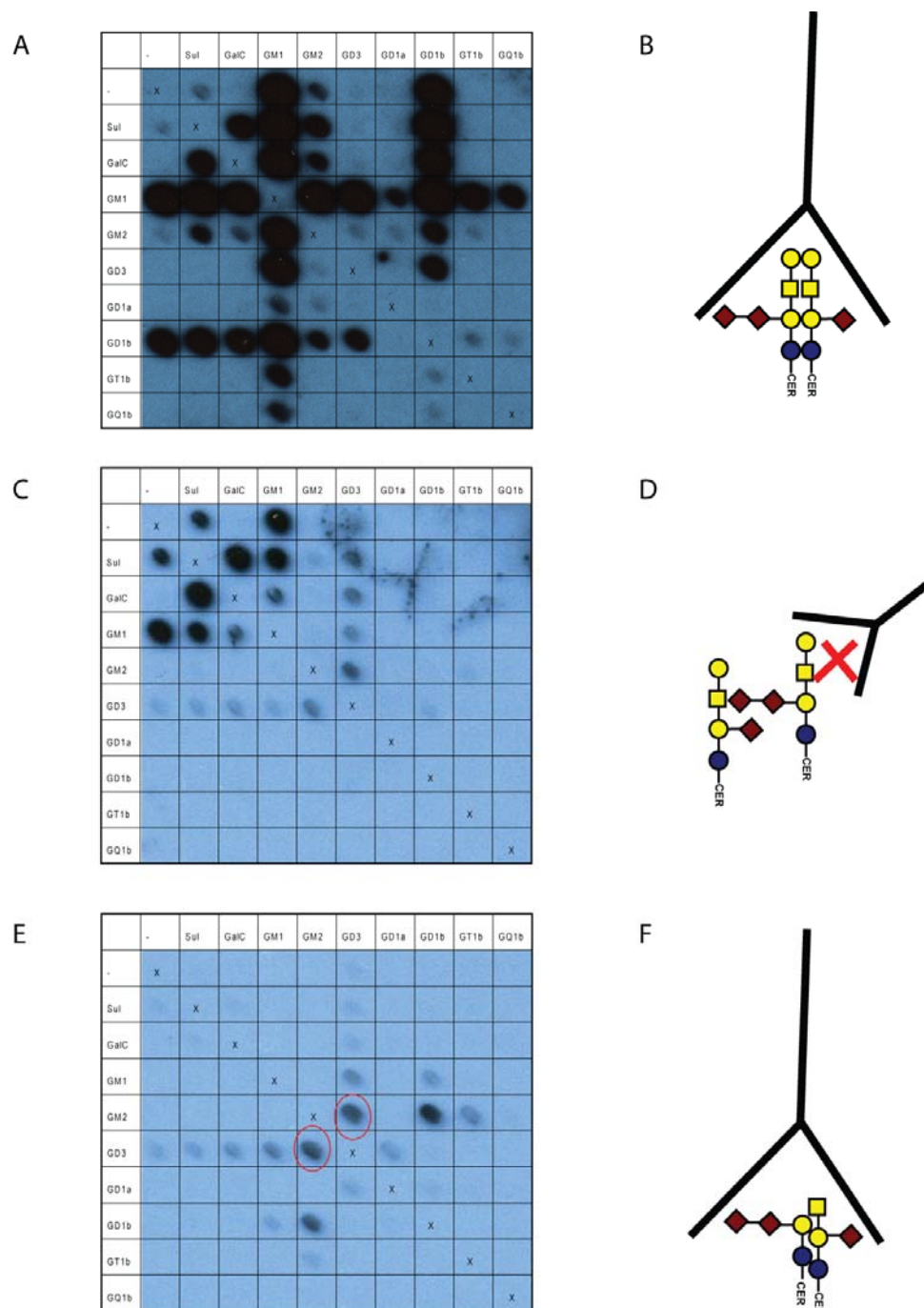
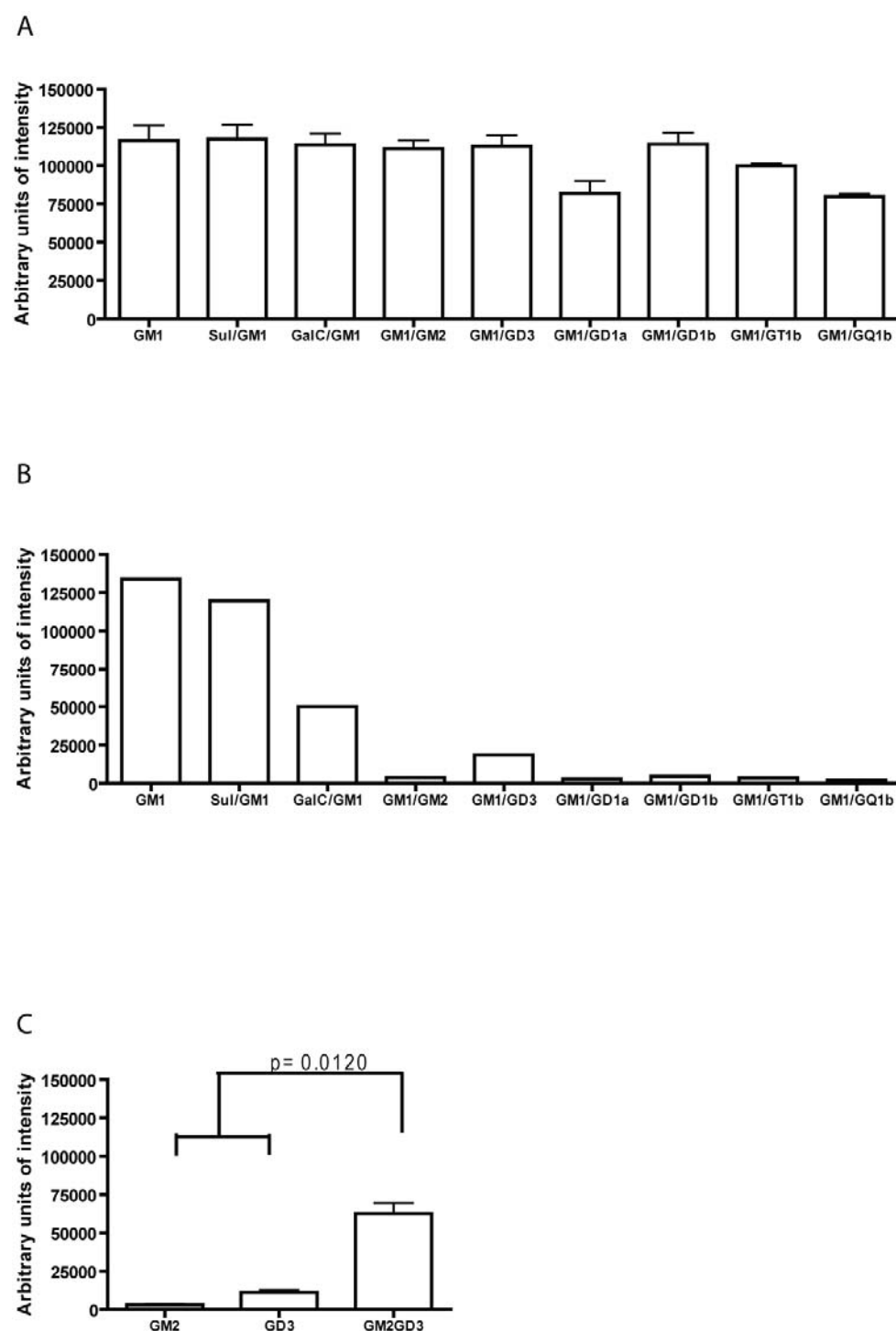


Figure 4.14 Patterns of AGA binding I

A & B. Serum 13; illustrative blot demonstrating “complex independent” binding of antibody to GM1 e.g. GM1:GD1b

C & D. Serum 91 illustrative blot demonstrating attenuation of anti-GM1 IgM when GM1 paired with more complex gangliosides e.g. GM1:GD1b

E & F. Serum 73 illustrative blot demonstrating enhanced binding to the complex created by GM2/GD3



**Figure 4.15. Patterns of AGA binding II.**

A. Bar chart of binding to GM1 alone and in complexes in serum 13. There is no significant difference in binding (Kruskal-Wallis with Dunn's correction,  $n=3$ )

B. Bar chart of binding to GM1 alone and in complexes in serum 91 ( $n=1$ )

C. Reactivity to complex of GM2/GD3 is increased in patient 73 compared to the sum of intensities for each contributing lipid partner ( $p=0.0120$ , 2 tailed t-test,  $n=3$ )

Table 4.6. ELISA and combinatorial blot specificity for AGA positive patients\*

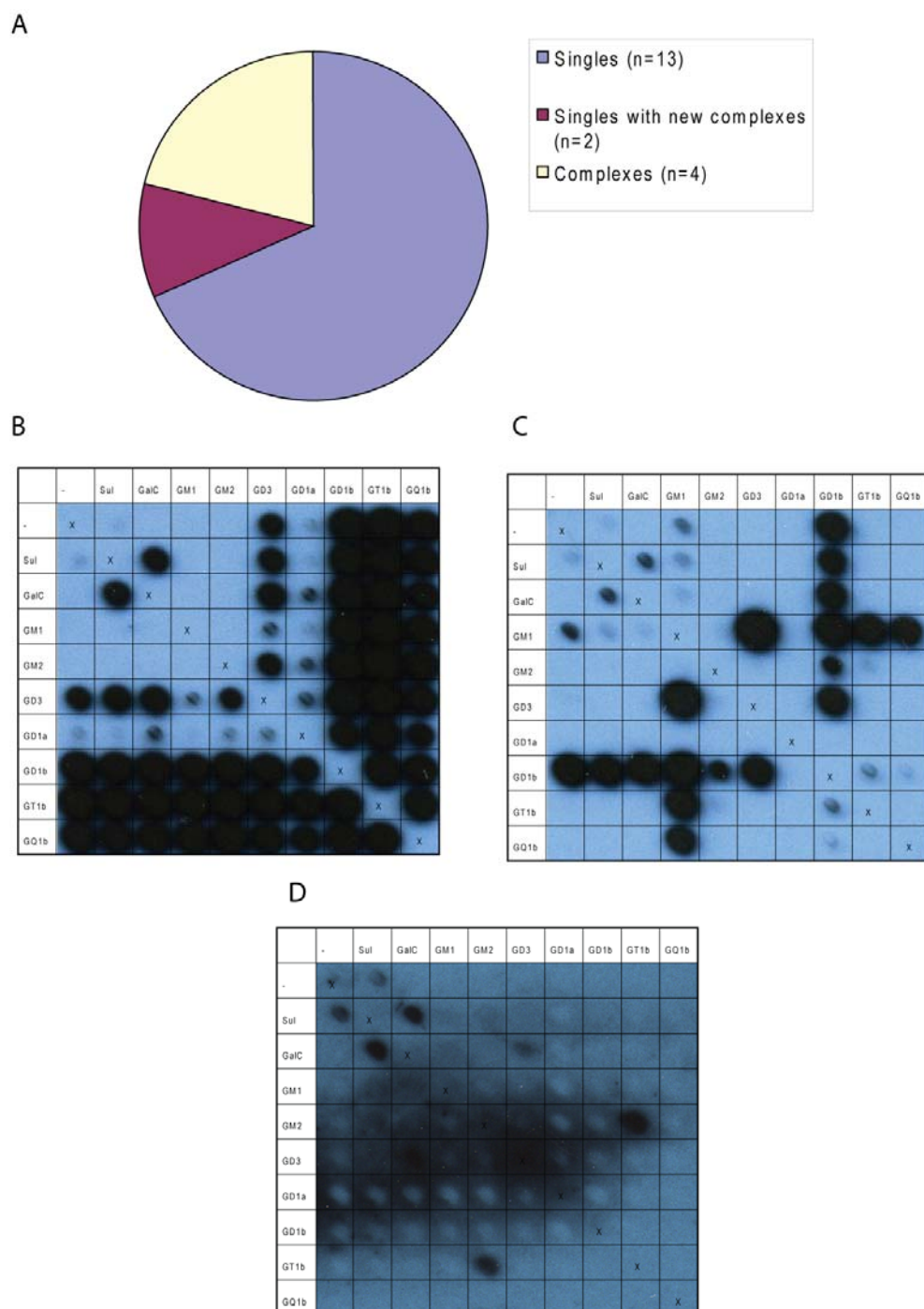
ID	ELISA specificity	Blot specificity
13	GM1, GA1, GD1b	GM1, GM2, GD1a
18	GM1	†GM1/-
26	GD1a, GT1b	GD1a, GT1b
28	GD1b	GD1b
31	GM1, GM2, GD3, GD1b, GT1b, GQ1b	GM2, GD3, GD1a, GD1b, GT1b
35	GM2, GT1b	Sulfatide & complexes
36	GM2	GM2
37	-	GD3
41	-	Complexes
43	-	Complexes
44	-	GM1
63	GD1a, GT1b	GD1a, GT1b
67	GM2, GD1a, GT1b	GM2, GD1a, GT1b
73	GD3	Complexes
75	GD1b	GM1, GD1b
79	-	Complexes
82	GD3, GD1a, GD1b, GT1b, GQ1b	GD3, GD1a, GD1b, GT1b, GQ1b
91	GM1	GM1, GD3
92	-	GM1

\*All ELISA positives are titres above 1 in 500. All combinatorial blot positives have arbitrary units of intensity >10000 and are associated with a positive spot on direct visual inspection.

† When first assayed this sample tested negative and was included in the heatmap data. However when the sample was re-assayed it bound GM1. This variability presumably is a reflection of the low titre (1 in 500 on ELISA).

Similarly to the GBS data we can divide the AGA positive CIDP patients into different groups depending on their glycolipid antibody binding patterns with 3 separate groups (see Figure 4.16). The majority (13/19) demonstrated binding to single gangliosides and to those gangliosides when combined with other lipids. 4/19 demonstrated binding only complexes of gangliosides.





**Figure 4.16. Patterns of AGA binding**

- A. Pie chart demonstrating IgM binding pattern of 19/57 CIDP sera
- B. Serum 82 demonstrating binding to single lipids and their complexes
- C. Serum 75 demonstrating binding to the single lipid GD1b and the complex created by GM1:GD3
- D. Serum 41 demonstrating binding to the complex GT1b:GM2



As per Figure 4.16 (C) in one patient there was evidence of enhanced IgM reactivity to the complex created by GD3 and GM1 (see Figure 4.17). Interestingly this patient had high titre antibody to GD1b. When the structures of all three molecules are investigated it may well be the case that GD3 and GM1 cluster together to mimic the shape of GD1b. Therefore this binding to a neocomplex may simply reflect the resilience of the “anti-GD1b antibody” to recognize its preferred antigen conformation no matter what the circumstance.

We identified 3 patients with IgM binding to GD1a and GT1b using ELISA and array methodology (Figure 4.18). As both gangliosides share a common structure moiety (Figure 4.19.), terminal NeuAca2-3GalB- moiety it is likely that this structure represents the antibody’s binding site in these particular patients. These patients were not clinically distinct compared to the rest of the CIDP cohort (see Table 4.7.).

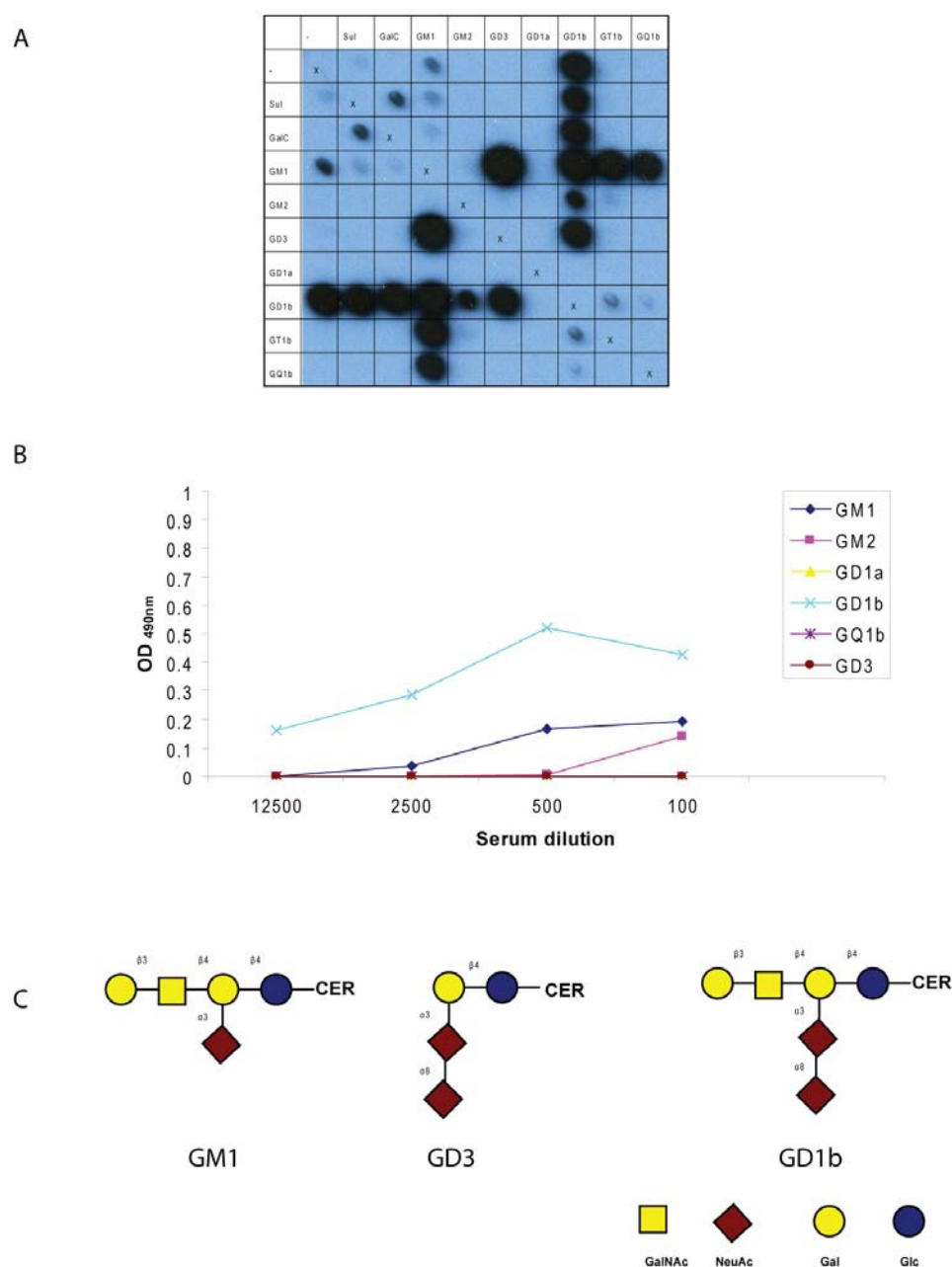


Figure 4.17 IgM GD1b AGA patient with reactivity to the complex created by GM1 and GD3.

A. Illustrative blot of serum 75

B. ELISA of serum 75 demonstrating binding to GD1b

C. Molecular structures of GM1, GD3 and GD1b

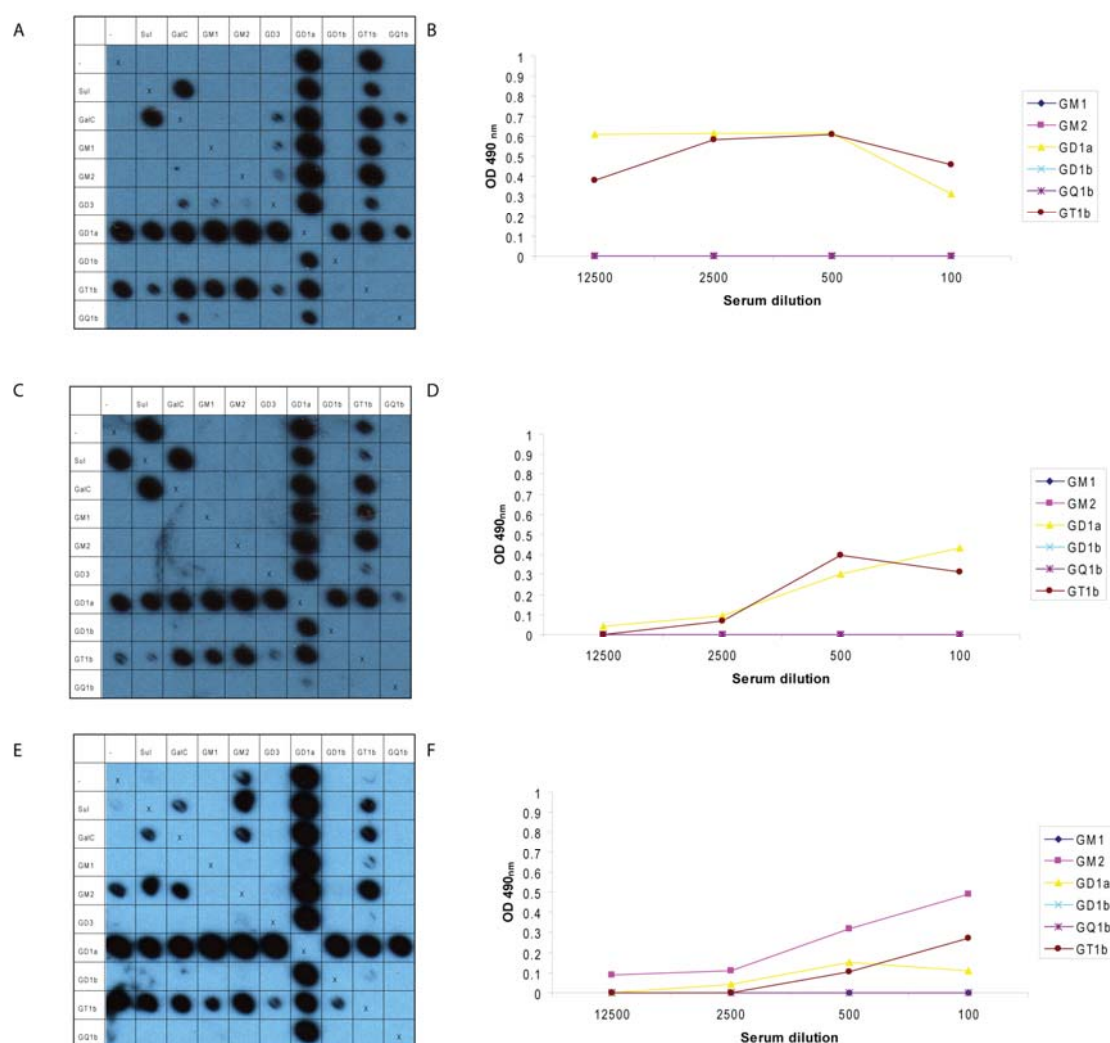
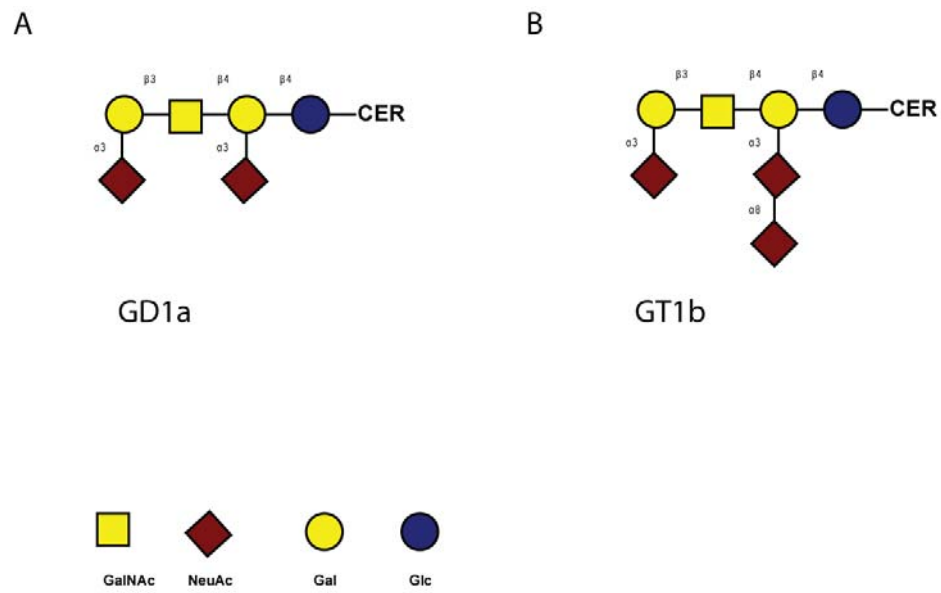


Figure 4.18 Array and ELISA data for GD1a/GT1b IgM positive patients

A & B represent illustrative blot & ELISA data for serum 26

C & D represent illustrative blot & ELISA data for serum 63

E & F represent illustrative blot & ELISA data for serum 67



**Figure 4.19. Structures of gangliosides GD1a and GT1b.**  
Abbreviations: GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; Gal, galactose; Glc, glucose; CER, ceramide

Table 4.7. Clinical characteristics of IgM GD1a/GT1b positive patients.

	26	63	67
Sex	Male	Female	Male
Age	64	67	38
Disease duration	11	3	14
MRC sum score (range 0-80)	48	63	73
ONLS (range 0-12)	6	2	4
UL ONLS (range 0-5)	2	0	2
LL ONLS (range 0-7)	4	2	2
Sensory score (range 0-64)	22	6	0
MCV (average)	36	35.5	45.5
dCMAP (average)	1.8	2.8	11.2

MRC sum score (possible range 0-80) higher values indicate greater strength;  
 Sensory sum score (possible range 0-64) higher values indicate greater  
 impairment.

#### 4.4. Discussion

13 of 57 CIDP patients demonstrated IgM binding to glycolipid antigens using the technique of ELISA. These sera displayed varying titres to a wide variety of glycolipid antigens from the simple GM1 ( $n=6$ ), GM2 ( $n=6$ ), asialo-GM1 (GA1) ( $n=7$ ), GD3 ( $n=4$ ) to the more complex gangliosides including GD1a ( $n=6$ ), GD1b ( $n=8$ ), GT1b ( $n=7$ ) and GQ1b ( $n=1$ ). 10/13 sera demonstrated binding to more than 1 glycolipid with 3 sera demonstrating anti-glycolipid antibody binding of a monospecific nature.

All samples testing positive via ELISA were similarly positive using the combinatorial array method. Although in some instances the ganglioside profile on ELISA did not exactly match that found using the array method. Use of the combinatorial array allowed the identification of an extra 6 patients demonstrating IgM reactivity to glycolipids. Using both techniques of ELISA and the combinatorial array, glycolipid reactivity did not segregate the CIDP patients into clinically or neurophysiologically distinct groups.

Using ELISA, IgM directed against GD1b was the only antibody seen specifically more frequently in the CIDP group compared to the combined groups of MS and HCs. These GD1b reactive patients did not differ clinically or electrophysiologically from other CIDP patients. Although it is noteworthy that these patients display a wide range of anti-GD1b titres. In addition they display reactivity to a wide range of other glycolipids on ELISA and array platforms (Table 4.2. and Table 4.6.). Often GD1b reactivity, in conjunction with reactivity to other disialylated gangliosides, is seen in

conjunction with an IgM paraproteinaemic neuropathy(Willison et al. 2001). The clinical picture here is characterized by preserved motor function in the limbs with a sensory ataxia. Interestingly one of the CIDP cohort, serum 82, which is illustrated in Figure 4.16 (A) displays strong binding to the gangliosides GD3, GD1b, GT1b and GQ1b using the array technique. This pattern of binding was observed on ELISA with additional strong reactivity to GD1a (see Table 4.2). GD3, GD1b, GT1b and GQ1b all share a common moiety the terminal NeuAc( $\alpha$ 2-8) NeuAc ( $\alpha$ 2-3) Gal which is likely to be the primary binding site for this IgM antibody. Furthermore this particular patient, who has had CIDP for 20 years, has minimal motor deficit scoring well with a total MRC score of 68/80 (higher scores indicate higher strength) with a high sensory sum score of 40/64 (higher scores indicate greater impairment).

In contrast to the ELISA method, using the combinatorial array did not reveal that any glycolipid/glycolipid complex antibody was demonstrated more frequently in the CIDP cohort.

Previous work involving animal models of CIDP have suggested that the simple lipid galactocerebroside, comprising a galactose molecule linked to a ceramide, is implicated in the pathogenesis of this disease. Chronic experimental autoimmune neuritis (the animal model of CIDP- EAN) has been shown to be induced in rabbits by immunisation with galactocerebroside(T. Saida et al. 1979). Here rabbits were immunized repeatedly with bovine brain galactocerebroside and developed a neurological disorder specific to the peripheral nervous system characterised by flaccid quadraparesis, limb hypaesthesia and respiratory paralysis. A subsequent study employing intraneural injection of the rabbit anti-galactocerebroside serum produced focal demyelinating lesions in rat sciatic nerves(K. Saida et al. 1979). A further study suggests that this anti-

galactocerebroside serum mediates pathology via antibody dependent mechanisms and complement activation as demyelinating activity was lost after complement inactivation (Sumner et al. 1982). However to date no group has reported an association of anti-galactocerebroside antibodies with CIDP patients (Hughes et al. 1984) (Rostami et al. 1987) (McCombe, Pollard & McLeod 1988a). This study agrees with these earlier reports of a lack of autoimmune response against galactocerebroside. In addition in keeping with observations from other groups there was no demonstrable IgG or IgM response to the closely related lipid, sulfatide, which was specific to the CIDP group.

Using ELISA I observed many different patterns of antibody specificity. In the case of anti-GM1 antibody specificity three main patterns have been described (Willison et al. 2002) :

1. Anti-GM1 antibodies that cross react with the terminal Gal (B1-3) GalNAc structure and will therefore react with those glycolipids bearing the same terminus such as asialo-GM1 and GD1b.
2. Anti-GM1 antibodies that cross react with GM2 as both lipids share an internal sialylated galactose moiety
3. Anti-GM1 antibodies that are monospecific to GM1 only

Six sera had demonstrable titres to GM1 (Table 4.2). In keeping with the first pattern described above, one of these sera bound the three glycolipids GM1, asialo GM1 and GD1b using ELISA. However when assayed using the combinatorial array binding was demonstrated to GM1, GM2 and GT1b (see



Figure 4.14 (A)). As asialo-GM1 was not employed in the array technique we cannot comment on binding to asialo-GM1 using this technique. No sera with specificity to GM1 and GM2 alone were observed. Two of the GM1 reactive sera reacted in a monospecific fashion to GM1 alone using ELISA but when the combinatorial blot was employed one of these sera recognized GD3 in addition to GM1. The remaining three GM1 positive sera bound to a number of other glycolipid antigens (between 4 and 7) when assayed by ELISA. In each of these three cases (see Table 4.2) reactivity for other glycolipid antigens was higher than that observed for GM1. These three sera also had differing profiles using the combinatorial blot with one not recognizing GM1 whatsoever, another only recognizing GM1 when complexed with other lipids, and the third recognizing GM1.

This variation in specificity between ELISA and the combinatorial blot is almost certainly a reflection of the platform upon which the lipids are presented. Compared to a plastic ELISA plate, PVDF is a porous substance and one may hypothesize that when lipids are presented using this platform they create conformations that are different to those created on an ELISA plate. Which technique more closely relates that of human myelin is as yet unclear.

However this possible variation in conformation clearly does not affect the findings in all sera as illustrated by the GD1a and GT1b reactive sera (illustrated in Figure 4.18) in whom findings on ELISA and the combinatorial blot method correlate well. Clearly neither technique of antibody detection is a perfect gold standard assay. We know that gangliosides aggregate in functional cholesterol rich microdomains with other glycolipids and proteins (Simons et al. 1997). The “gold standard” assay will need to take this into consideration.

These patients with demonstrable IgM responses to GD1a and GT1b (figure 4.18) did not differ clinically nor electrophysiologically from other antibody positive or negative patients (Table 4.4). As GD1a and GT1b share the same terminal NeuAc ( $\alpha$ 2-3) Gal moiety it is likely that this IgM antibody recognizes this particular conformation (Figure 4.19). Both gangliosides serve as ligands for myelin associated glycoprotein (MAG)(Collins et al. 1997). MAG is located in the periaxonal membrane of Schwann cells(Trapp et al. 1989). Mizutani and colleagues reported a male patient with a demyelinating neuropathy with a similar antigen specificity of IgM to GM3, GD1a and GT1b(Mizutani et al. 2001).The authors make the observation that the neuropathology observed in mice deficient in such complex gangliosides is similar to that seen in MAG deficient mice(Sheikh et al. 1999). They then speculate that in their case antibodies to the glycolipid antigens could potentially play a role in a demyelinating process by inhibiting adhesion between myelin and axonal membrane. Interestingly when reassayed on a wider panel of lipids only one of these sera bound to the closely related lipid GM3 which shares the same terminal epitope. In addition a MAG IgM was not demonstrated in any of these samples. In fact the only positive serum for MAG bound the structurally different glycolipid GM2 using both ELISA and the array method.

Work into the responses of CIDP serum to glycolipid complexes is underway across many research groups. One study has recently investigated anti-complex antibodies in CIDP and MMN (multifocal motor neuropathy) using ELISA. They investigated sera IgM reactivity to 5 gangliosides (GM1, GM2, GD1a, GD1b and GT1b) and all their 1:1 combinations(Nobile-Orazio et al. 2010). New reactivities to complexes were only seen in patients testing positive for other gangliosides. Modulation of lipid binding was apparent in

the MMN group with binding to GM1 attenuated by GD1a. In contrast to Nobile-Orazio's work we did demonstrate 4 patients with new reactivities to complexes with no demonstrable binding to single glycolipids. However these complex reactive sera compiled a small subset of the glycolipid reactive sera (4/19). This contrasts with our GBS data where we observed a higher proportion of patients (15/42) with detectable antibody only to complexes of lipid with 26/42 in total demonstrating anti-ganglioside complex reactivity.

The small number of glycolipid reactive sera (19/57) may be a consequence of the number lipids employed in the combinatorial array. With the current technique we are limited in the number of glycolipid antigens we can print per slide and did not include such lipids as LM1 (sialosylneolactotetraosylceramide) and SGPG (sulfated glucuronyl paragloboside). Both lipids are difficult to isolate and are not commercially available. LM1 is a major peripheral nerve myelin glycolipid and its structure is closely related to that of GM1 (Ogawa-Goto et al. 1998). However one early study has not demonstrated any IgM response to this lipid in a CIDP cohort (Melendez-Vasquez et al. 1997). SGPG has a similar structure to LM1, except for a 3-sulfated glucuronic acid instead of a sialic acid on the terminal saccharide chain. Initial investigations into responses against this lipid in CIDP did not prove fruitful however in 1996 Yuki et al demonstrated IgM response to SGPG in 12/30 (40%) CIDP patients (Ilyas et al. 1991) (Ilyas, Mithen, Dalakas, Chen & Cook 1992a) (Yuki, Tagawa & Handa 1996a). Including these lipids in future combinatorial arrays may yield a higher number of glycolipid reactive CIDP sera. However an alternative explanation is that this small number of lipid reactive IgM are simply an epiphenomenon of other pathological cascades and that anti-glycolipid antibodies do not have a significant role in CIDP.

Antibody positive patients did not differ clinically or neurophysiologically from antibody negative patients which may suggest that we are continuing to search for the wrong antigens. The failure of this combinatorial approach to recognize a common antigenic target in CIDP may reflect the heterogeneous clinical and perhaps, the pathological nature of CIDP. If specific subsets of CIDP patients are interrogated (e.g. those who respond well to immunotherapies) for anti-glycolipid responses using a wider range of glycolipids, we may have success in the search for the elusive target antigens involved in this disease.

## Chapter 5. Multiple Sclerosis

### 5.1. Introduction

The realization that lipids exist in functionally distinct rafts in partnership with fellow lipids and the recent description of anti-ganglioside complex antibodies leads us to question whether anti-lipid complex antibodies are a feature of multiple sclerosis. Our first series of analyses was to use the combinatorial lipid array approach to investigate antibody specificities of multiple sclerosis CSF.

We wanted to investigate the response of MS CSF to central nervous system myelin derived lipids. Myelin is particularly characterized by the high ratio of lipid to protein. Produced by oligodendrocytes within the CNS, myelin typically is composed of 70-85% lipid and 15-30% protein(Norton 1977)(Anderson et al. 1992). Table 5.1. demonstrates Norton's findings of composition of central nervous system myelin. The 3 main groups of lipids found in myelin include cholesterol, phospholipids and galactolipids. There are no lipids which are specific to human myelin however it is characterized by relatively high levels of galactocerebroside. In addition to the two main galactolipids, galactocerebroside and sulfatide, myelin also contains some minor galactolipids including fatty acid esters of galactocerebroside and the glycerol based lipids, mono and digalactosyldiglyceride(Norton 1977). Gangliosides have also been reported in myelin in particular GM1 accounting for 70 moles % of total ganglioside. The galactosylceramide derived ganglioside GM4 is also present in CNS myelin. An additional factor in the heterogeneity of these myelin based lipids includes the length of the fatty acid chain, degree of hydroxylation and degree of saturation. Oligodendrocytes synthesize galactolipids that characteristically contain in their ceramide part very long chain fatty acids with 18 to 24 carbon atoms

Table 5.1. Composition of human central nervous system myelin- adapted from Table 1, Chapter 5, Isolation and Characterization of Myelin, Myelin, 2<sup>nd</sup> Edition(Norton 1977).

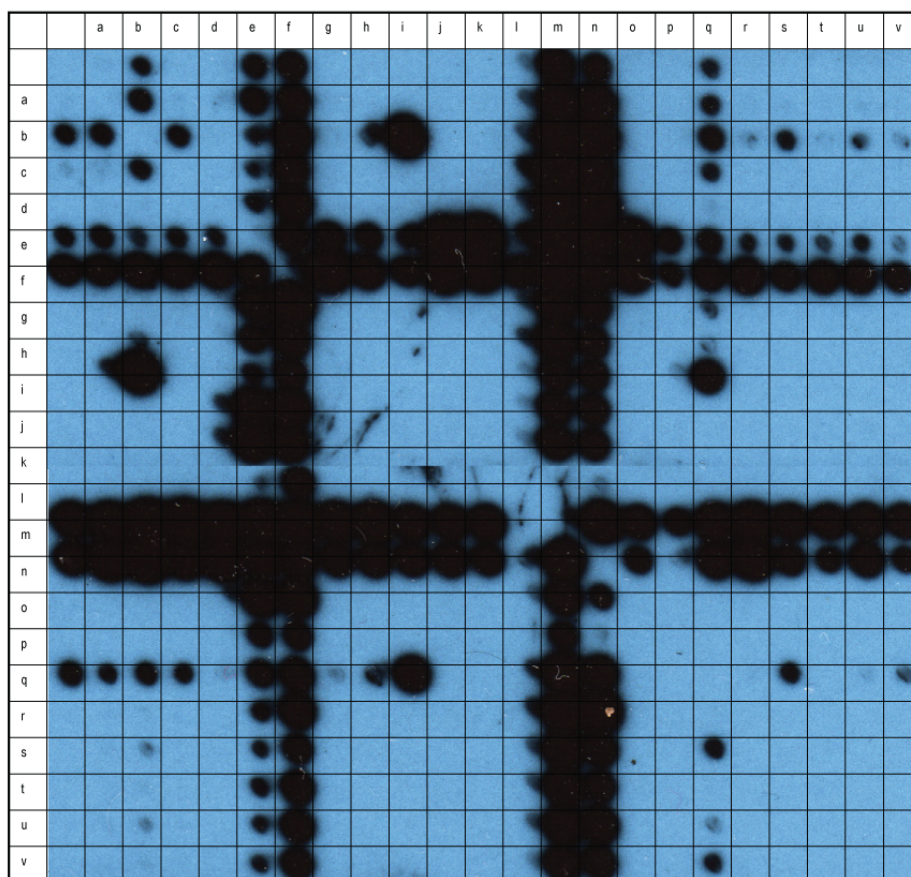
Substance	Human myelin
Total protein <sup>a</sup>	30
Total lipid <sup>a</sup>	70
Cholesterol	27.7
Total galactolipid	27.5
-galactocerebroside	22.7
-sulfatide	3.8
Total phospholipid	43.1
- ethanolamine phosphoglycerides	15.6
-choline phosphoglycerides	11.2
- serine phosphoglycerides	4.8
- inositol phosphoglycerides	0.6
-sphingomyelin	7.9
Plasmalogen	12.3

<sup>a</sup> Protein and lipid figures are in percentage dry weight; all others are in percentage total lipid weight

We started with a large scale array of 22 commercially available lipids (Table 2.1 and Figure 5.1.) that represented lipids that were constituents of CNS myelin and that were commercially available. Details of lipid origin are outlined in Chapter 2, Table 2.1. We screened CSF derived from MS patients (n=12) and patients with other neurological disease (OND) (n=12). No binding to gangliosides was observed in any of the MS patients however a there was a non statistical trend observed for binding to the myelin derived lipid sulfatide. Therefore a smaller combinatorial grid was composed consisting of predominantly myelin derived lipids.



A



B

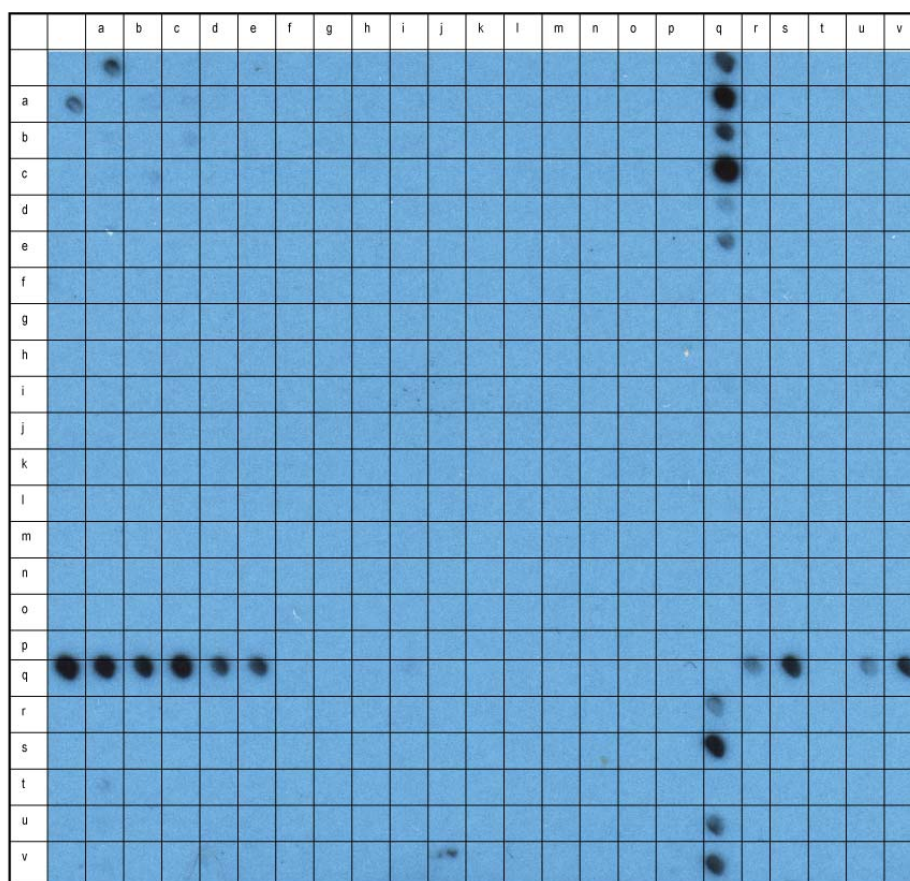




Figure 5.1. Preliminary combinatorial grid.

Key to the grid is outlined below. Row and column headings reveal the complex at each location

A. Serum 070536

B. CSF from MS patient 20630

A	Sulf	l	GD1a
B	GalC	m	GD1b
C	Chol	n	GT1a
D	Sphingomyelin	o	GT1b
E	GA1	p	GQ1b
F	GM1	q	DGG
G	GM2	r	PC
H	GM3	s	PE*
I	GM4	t	PIP (4)
J	GD2	u	PL
K	GD3	v	PS

The final 10 candidate lipids were used in the array included:

1. Sulfatide; this was a lipid of interest due to the description of antibodies reactive to sulfatide within MS CSF as discussed earlier.
2. Galactocerebroside; this closely related lipid has similarly been investigated in MS. Various *in vitro* models have suggested that anti-galactocerebroside antibody may have demyelinating characteristics(Fry et al. 1974)(Raine et al. 1981). Fry et al demonstrated that, when applied to mouse spinal cord cultures, antisera to galactocerebroside inhibited sulfatide synthesis and led to demyelination. Later Raine et al demonstrated that the demyelinating capabilities of anti-galactocerebroside sera on mouse spinal cord cultures was comparable to that observed with sera against whole white matter.

Also passive antibody transfers in myelin basic protein- primed animals further suggest the demyelinating properties of anti-galactocerebroside antibodies(Fierz et al. 1988). Further work suggests that anti galactocerebroside antibodies and not anti sulfatide antibodies alter the organization of myelin membrane sheets in both the PNS and CNS(Dyer et al. 1988). Both galactocerebroside and sulfatide have been implicated in the mediation of  $\text{Ca}^{2+}$  influx with each lipid mediating this function independently(Dyer et al. 1991). It has been suggested therefore that galactocerebroside and sulfatide may play different roles in the regulation and maintenance of myelination with antibody binding disrupting this process(Stoffel et al. 1997). In relation to CNS demyelination a recent study suggests that serum anti-galactocerebroside antibodies may be MS specific and may further aid in disease stratification being observed more frequently in the relapsing-remitting subgroup(Menge et al. 2005).

3. Ceramide was chosen because it is the natural precursor lipid of the galactolipids galactocerebroside and sulfatide (Chapter 1, Figure 1.5.)(Stoffel et al. 1997).
4. Cardiolipin was chosen as a “reference” lipid not typically seen in CNS myelin.
5. Sphingosine was chosen as it is a precursor of galactolipids.
6. Sphingomyelin was chosen as it is highly expressed in CNS myelin (Table 5.1.).
7. Cholesterol is the major contributory lipid to myelin and was therefore included.
- 8 & 9. Galactosyl diglycerides occur in brain tissue. Monogalactosyl diglyceride is transformed into digalactosyl diglyceride by  $\alpha$  galactose. The enzymes involved in the synthesis of these two molecules are present in oligodendrocytes and their activity appears to be related to the process of myelination during development(Pieringer et al. 1973).Early work by Hirsch and colleagues has suggested that MS derived sera is particularly reactive to the lipid, digalactosyl diglyceride, demonstrating that MS sera caused lysis preferentially of liposomes created with digalactosyl diglyceride(Hirsch et al. 1976).
10. As there was only one place left on the combinatorial grid one phospholipid was chosen to complement the selection of lipids. No one phospholipid appeared to affect binding in any significant manner on the initial array therefore one, phosphatidylcholine, was chosen randomly from the group of available phospholipids.

## 5.2. Methods

### 5.2.1. ELISA

The specificities of sera for gangliosides were assayed by ganglioside ELISA as described in section 2.2.3. A positive binding result was considered to give an OD<sub>490nm</sub> of 0.1 or above.

### 5.2.2. Combinatorial array

Anti-complex lipid reactivity was assayed by combinatorial lipid array as described in 2.2.4. We investigated CSF and serum IgG response to 10 individual lipids (see Table 2.1. for details): sulfatide (Sulf) , galactocerebroside (GalC) , ceramide (Cer), cardiolipin (Cardio) (Sigma-Aldrich, UK), sphingosine (SS), sphingomyelin (SM), cholesterol (Chol), digalactosyl diglyceride (DGG) , monogalactosyl diglyceride (MGG) (Sigma-Aldrich, UK), L alphaphosphatidylcholine (PC) and all their possible 1:1 complexes (55 target antigens in total). CSF and serum samples were probed at 1:10 and 1:100 dilutions respectively. Monoclonal antibodies were probed at a concentration of 10µg/mL unless otherwise stated. When testing the recombinant Fab-fragments membranes were probed as before. However instead of using anti-human IgG as a secondary, 1:5000 diluted horseradish peroxidase-labelled anti-HIS (C-terminal) antibody (Miltenyi) was employed. A chemiluminescent reaction was employed again using ECL plus (Amersham/GE Healthcare, UK) and rendered on radiographic film after 1 minute exposure. Films were digitalized by flatbed scanning and the images quantified by Total Lab software (Amersham Biosciences, UK).

### 5.2.3. Statistical analysis

ANOVA analyses were used to compare anti-glycolipid levels between more than three groups. The Mann-Whitney test was used to compare anti-

glycolipid antibody levels between two groups. Paired t tests were used to detect if anti-complex antibodies were of higher intensity than antibodies to the contributing single lipid. Differences in proportions were tested for significance by chi-squared test and Fisher's exact test where appropriate. These analyses were performed with PRISM4 (GraphPad software, USA). Raw data was logarithmically transformed (log 2) and heatmaps, hierarchical clustering and further ANOVA analyses were performed by Dr Gabriela Kalna of the University of Glasgow using Partek Geometrics Suite software (Partek Incorporated, USA).

#### 5.2.4. Immunohistology of Myelinating cultures

In vitro myelinating cultures were established according to Sorenson et al and were carried out by Dr Ariel Arthur, University of Glasgow (Sorensen et al. 2008). Briefly, a single cell suspension was prepared from E15.5 rat spinal cord (Sprague Dawley) and plated on to a confluent monolayer of neurosphere derived astrocytes in 50% DMEM, 25% horse serum (heat inactivated), 25% HBSS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , and 2 mM L-glutamine (Invitrogen) (plating media) at a density of 150,000 cells/200 $\mu\text{L}$ /13mm diameter cover slip. Cells were left to attach for 2 hrs at 37°C after which was added an additional 300 $\mu\text{L}$  of plating media and 500 $\mu\text{L}$  of differentiation medium (DM) which contained DMEM (4,500 mg/mL glucose), 10ng/mL biotin, 0.5% N1 hormone mixture (1mg/mL apotransferrin, 20mM putrescine, 4 $\mu\text{M}$  progesterone, and 6  $\mu\text{M}$  selenium) 50nM hydrocortisone, and 0.5mg/mL insulin (Sigma). Cultures were maintained at 37°C/7%CO<sub>2</sub> and fed three times a week by replacing half the culture medium with fresh differentiation media. After twelve days in vitro (DIV) insulin was omitted from the culture medium to promote myelination.

Immunohistological interrogation of cultures (28 DIV) for the recombinant antibody binding (n = 8, Denver clones 4, 17, 33, 37, 73, 76, 80, 97) (Table 5.7) was performed as follows. Human antibodies, or the mouse IgM anti-sulfatide antibody, O4 were applied for 30 min at 4°C, in conjunction with the anti-MOG antibody, Z2. After washing in ice cold DMEM, cultures were fixed in 4% paraformaldehyde for 15 min at room temperature followed by exposure to the appropriate secondary antibody for 45 min at room temperature (Alexa Fluor®, Invitrogen). Unbound secondary antibody was removed by washing with PBS followed by distilled H<sub>2</sub>O and mounted in Vectashield (Vector laboratories).

#### 5.2.5. Patients

CSF and serum IgG lipid reactivities were investigated in archived MS and other neurological disease (OND) samples. In the group of 40 MS patients, 24 had relapsing-remitting MS (RRMS) and 16 had primary progressive (PPMS). All patients met the McDonald criteria for the diagnosis of MS (Polman et al. 2005). The group of 40 patients with OND had the following diagnoses: Neuropathy (9- including 1 GBS, 1 MMN and 2 chronic demyelinating neuropathies); Migraine (4); Idiopathic intracranial hypertension (4); Non-specific headaches (2); Negative investigations for demyelination (11); Parkinson's disease (2); Stroke (2); Cerebral venous sinus thrombosis (1); Viral meningitis (1); Cervical spondylosis (1); Dementia (1); Progressive ataxia (1); Pancreatic cancer (1). Casenotes were retrospectively reviewed for demographic data. Characteristics of the patients are outlined in Table 5.2. The study was carried out in accordance with the local ethics committee (South Glasgow and Clyde REC).

Table 5.2. Characteristics of MS patients

Variable	MS	OND
Number	40	40
Sex		
Male	26	12
Female	24	28
Age (y)		
Median	38.5	44.5
Range	25-64	16-69
CSF IEF		
isolated OCB	37	0
no bands	1	36
other*	2	4

\* Polyclonal or monoclonal CSF IgG or paired bands in CSF & serum

## 5.3. Results

### 5.3.1. CSF and serum from Scottish MS patients

Heat map analysis identified a disease-associated lipid-specific CSF antibody response in MS (Figure 5.2.) that preferentially targeted sulfatide (column 1, ANOVA with Bonferroni p-value adjusted for multiple testing;  $p=0.004$ ) and heterodimeric complexes of sulfatide with other lipids, including galactocerebroside ( $p=0.03$ ) (Figure 5.3.). Within individual CSF samples it was evident that antibody binding was modulated by specific lipid partnerships. In particular within MS CSF, binding to sulfatide was inhibited by combination with sphingomyelin ( $p < 0.01$ , 1 way ANOVA with Dunnett's correction) (Figure 5.2. (A) & Figure 5.3. (B)). Lipid reactive antibodies were detected in 60% of MS and 25% OND CSF. The majority (75%) of the lipid reactive MS CSF samples recognized sulfatide and/or sulfatide containing complexes. The CSF sulfatide binding in MS patients appears to be MS specific as it was mostly unaccompanied (77%) by a corresponding serum response to sulfatide or sulfatide containing complexes indicating the response was intrathecal ( $p=0.007$  Fishers exact test compared to OND CSF/serum pairs) and may therefore represent OCB specificities.



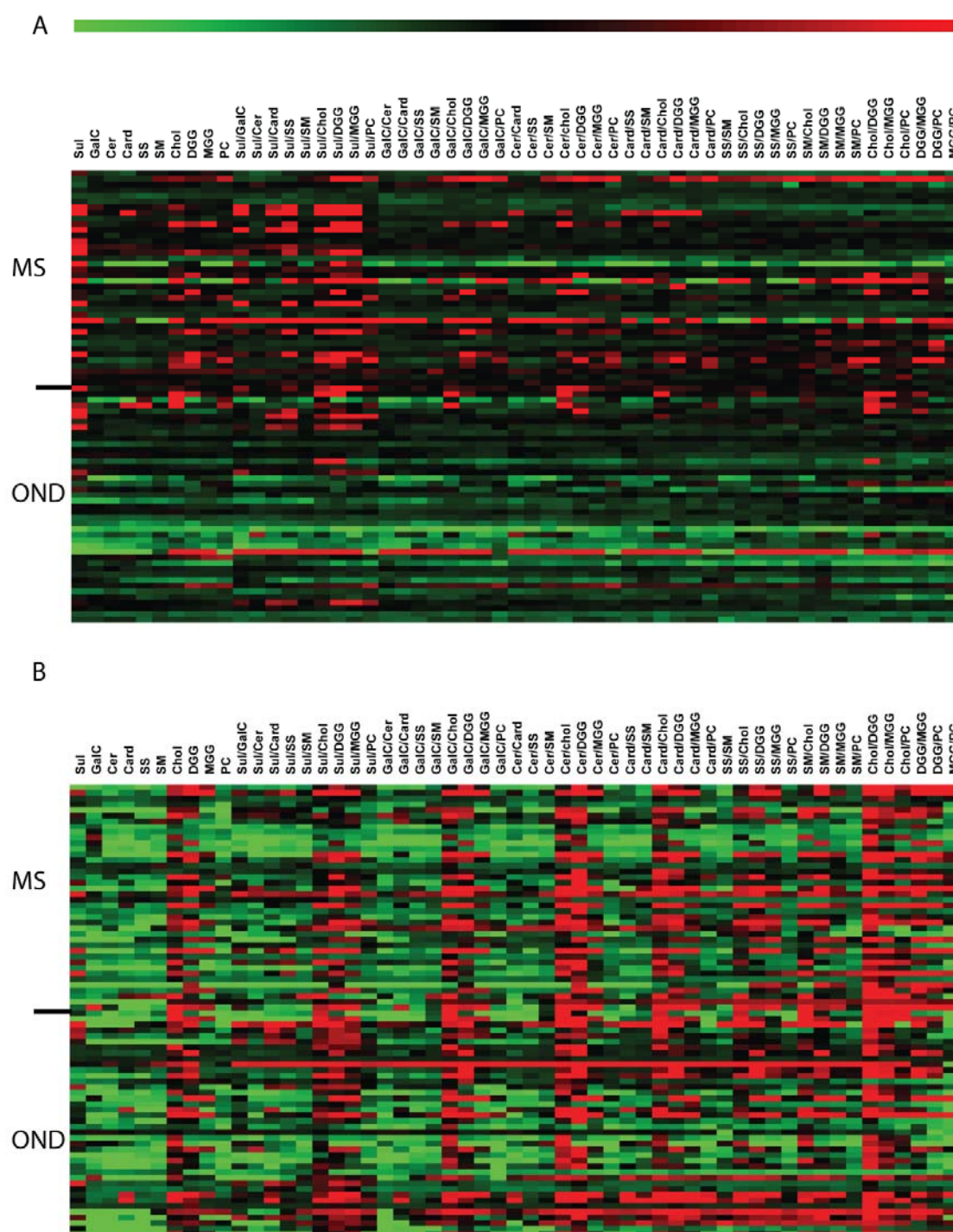
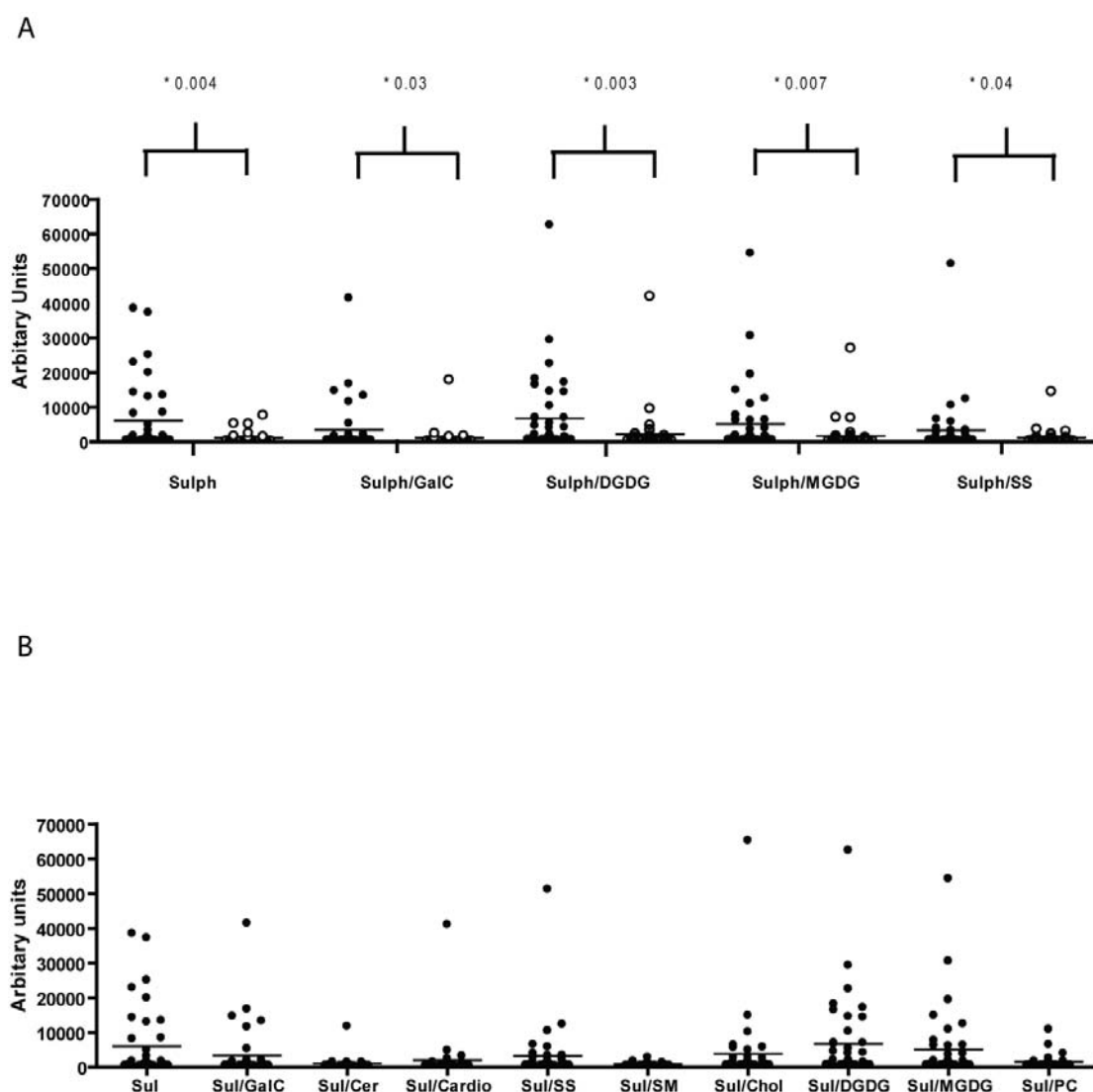


Figure 5.2. Array analyses in patient CSF and serum

Heatmaps, created using logarithmic transformations of the mean intensities recorded for each lipid antigen, depicting CSF (A) and serum (B) IgG reactivity in MS and OND patients. Lipids are displayed as column headings and each row represents an individual patient.



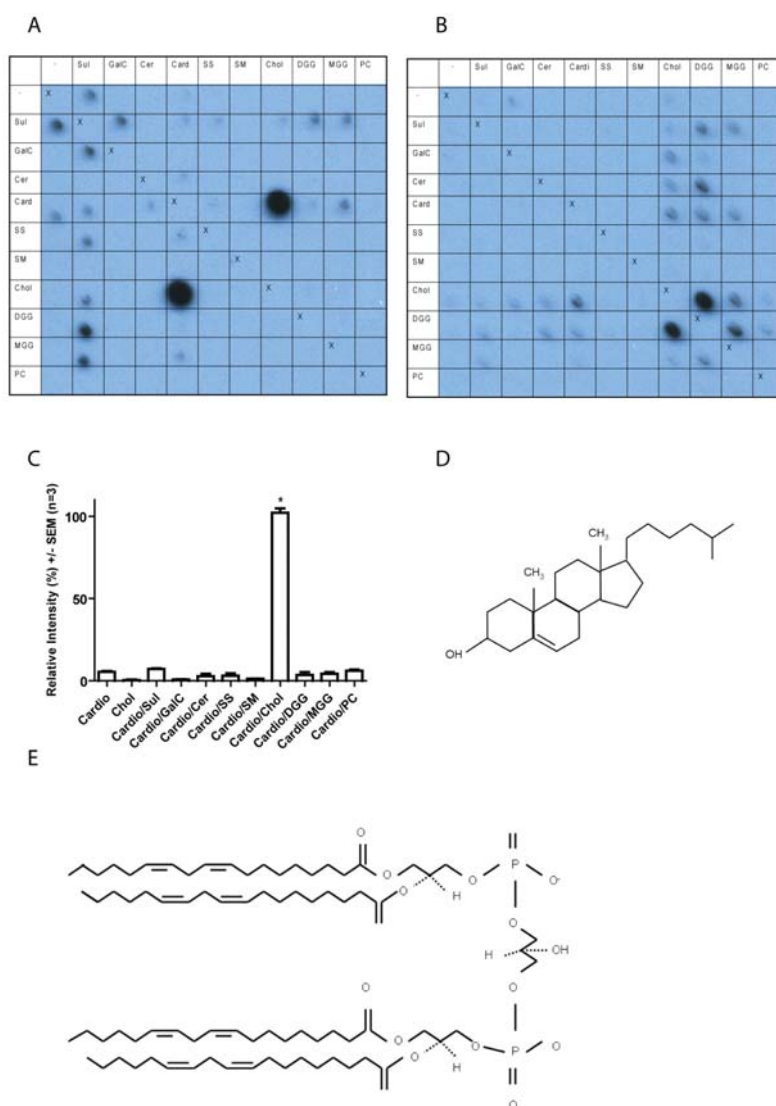
**Figure 5.3. Dotblots of anti-sulfatide IgG in CSF of MS & OND patients**

A. CSF anti-sulfatide/sulfatide complexes in CSF of patients with MS and OND. Closed circles denote MS CSF and open circles denote OND CSF. Reactivities to sulfatide and particular combinations of sulfatide with other lipids were observed more frequently within MS CSF. (p values shown have been obtained with ANOVA with Bonferroni).

B. CSF anti-sulfatide/sulfatide complexes in CSF of MS patients only. Binding to sulfatide is inhibited when combined with sphingomyelin ( $p < 0.01$ , 1 way ANOVA with Dunnett's correction)

Antibody binding was modulated strongly by interactions with other lipids resulting in different patterns of reactivity. These included both complex-enhanced recognition, in which antibody binding to a heterodimeric complex is greater than to either individual component, and complex-inhibited, in which certain lipids e.g. sphingomyelin inhibited antibody binding. Figure 5.4. illustrates complex enhanced recognition in MS CSF, where binding to the complex formed by cholesterol and cardiolipin is increased by 96.3% compared to the sum of the intensities for the individual lipids ( $p=0.0004$ , paired t test,  $n=3$ )

Figure 5.5. demonstrates complex inhibition. Here the CSF specific IgG binds to sulfatide to yield a mean relative intensity signal of 57.1%, yet the intensity for the complexes of sulfatide created with the lipids ceramide, sphingomyelin and phosphatidylcholine is almost eliminated at between 1.96% and 3.66% ( $p<0.0001$ , GLM ANOVA with Tukey,  $n=3$ ). This is in contrast to the corresponding serum sample from the same patient which demonstrates IgG reactivity to digalactosyl diglyceride which is complex independent i.e. binding is not modulated by lipid partnerships.



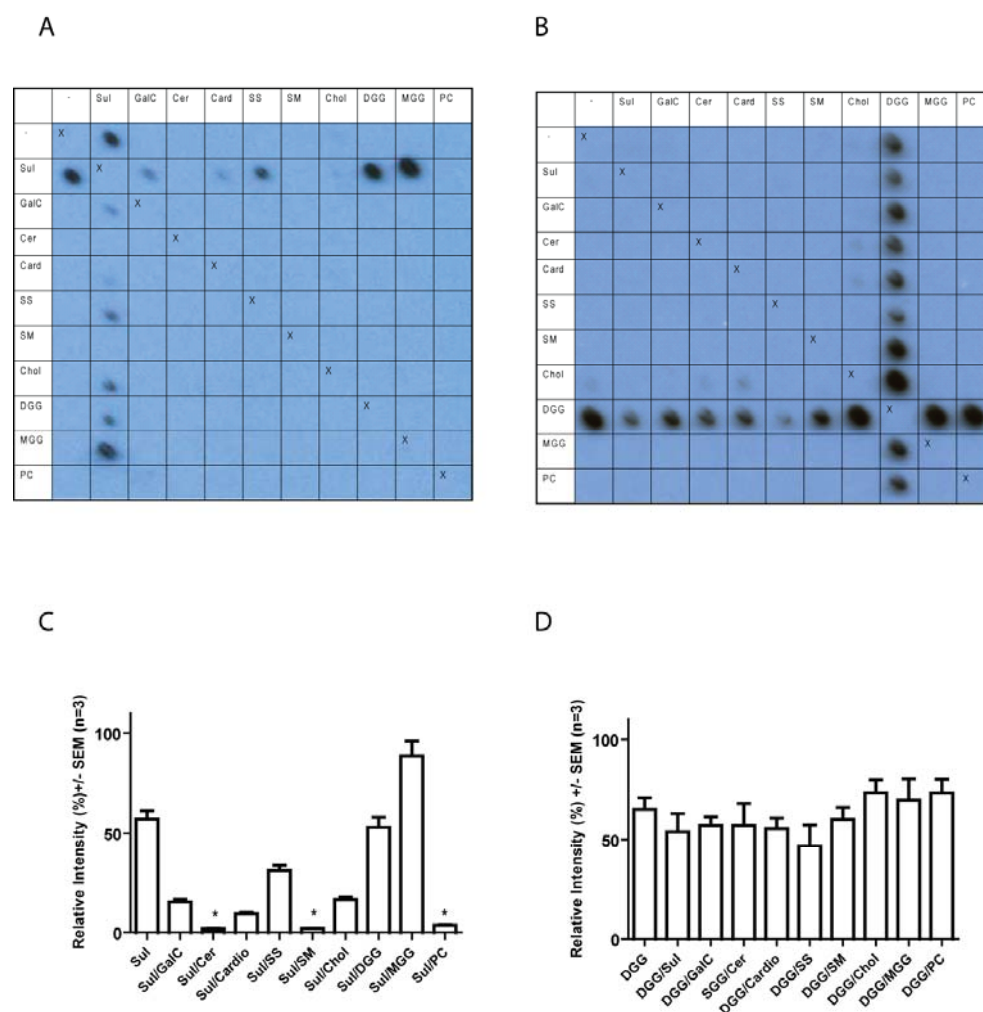
**Figure 5.4. Complex enhanced reactivity**

A. CSF IgG blot from MS patient ID 21699. Row and column headings reveal the complex at each location. "x"s represent the negative controls (methanol) which act as a line of symmetry for duplicate spots within the same membrane.

B. Serum IgG blot from MS patient ID 21699

C. Quantitative analyses of CSF IgG reactivity. The array units have been normalized with the highest observed value for CSF set at 100%. All other data is expressed as a percentage of this observed value.

D & E Molecular structure of cholesterol and cardiolipin respectively



**Figure 5.5. Modulation of lipid binding by lipid partnerships**

A CSF IgG blot from MS patient ID 40584

B Serum IgG blot from MS patient ID 40585

C Quantitative analyses of CSF 40584 IgG reactivity.

D Quantitative analyses of Serum 40585 IgG reactivity.

† The data in C & D have been normalized to the highest value observed in the CSF and serum datasets respectively.

This CSF IgG anti-sulfatide response appears to be restricted to the CSF as in the majority of MS patients there was no accompanying serum anti-sulfatide response. This is in contrast to OND samples where anti-sulfatide IgG was typically present in paired samples of CSF and serum ( $p=0.007$  Fishers exact test). One may assume then that these CSF specific IgG responses are intrathecal and may represent OCB specificities. However, whole CSF samples include antibodies from the polyclonal background (which predominantly originate from plasma filtered in the choroids plexus) in addition to the OCB therefore the anti-lipid response observed here may not be secondary to the OCB.

To distinguish individual OCB from irrelevant background antibodies, we collaborated with two independent research groups who developed separate techniques for isolating OCB. For the first approach recombinant antibodies were produced from single B- and plasma cells (Owens et al. 2009). This approach became feasible since it was shown that CSF-resident cells of the B lineage may produce OCB (Obermeier et al. 2008).

### 5.3.2. Lipid reactivity of recombinant antibodies from single CSF B cells

We investigated 100 recombinant antibodies (rAb) derived from clonally expanded CSF B cells/plasmablasts obtained from patients with MS ( $n=11$ ; 73 rAb) and other CNS inflammatory diseases (OIND;  $n=6$ ; 27 rAb) (Table 5.6) (Burgoon et al. 1999) (Owens et al. 2006) (Burgoon et al. 2006) (Bennett et al. 2009). Overall, lipid specific rAb were generated from 45% of MS patients and accounted for 27 % (20/73) of the total rAb, of which 19% (14/73) bound sulfatide and/or sulfatide containing complexes whilst the remaining 8% (6/73) recognized cholesterol (Figure 5.6.(A) and (B))

Antibody binding was strongly modulated by interactions with other lipids resulting in patterns of lipid reactivity similar to that observed in the whole patient CSF. This included both complex-enhanced recognition, in which binding to a heterodimeric complex is greater than to either individual component (Figure 5.7.A,C) and complex inhibited, in which certain lipids inhibit antibody binding (Figure 5.7. B, D). Even in the case of apparently complex independent responses (Figure 5.9) lowering the rAb concentration reveals the preferred target is provided by a sulfatide/galactocerebroside complex.

Table 5.3. Features of recombinant antibodies (rAbs) derived from MS and OIND CSF

Patient	rAb	Diagnosis	No of rAbs produced	No of rAbs reactive to lipid
MS 02-19	1, 2, 19, 20, 57, 58, 59, 60, 61	PPMS	9	0
MS 03-1A	3, 4, 5, 6, 69, 70, 71, 72, 73, 74, 75, 76, 77	MS	13	7
MS 04-2	7, 8, 9, 62, 63, 64, 65	PPMS	7	3
MS 03-7	66, 67	RRMS	2	0
MS 04-3A	68	RRMS	1	0
ON 03-5A	27, 28, 29 30	MS	4	0
ON 03-3A	21, 22, 23, 24, 25, 26	MS	6	0
ON 04-7A	31, 32, 33, 34, 35, 36, 37	MS	7	2
ON 04-8A	38, 39, 40, 41	MS	4	0
ON 07-7A	51, 52, 53, 54, 55, 56	MS	6	1
MS 05-3	10, 11, 12, 13, 14, 15, 16, 17, 18, 78, 79, 80, 81, 82,	RRMS	14	7
IC 05-2B	87, 88, 89, 90, 91, 92	Chronic meningitis	6	0
IC 06-1C	83, 84, 85, 86	SSPE	4	2
SSPE 83D	93, 94	SSPE	2	1
IC 04-4	99, 100	Cryptooccal meningitis	2	0
ON 07-5E	42, 43, 44, 45, 46, 47, 48, 49, 50	NMO	9	4
IC 08-5F	95, 96, 97, 98	VZV radiculopathy	4	3

<sup>A</sup> Single-cell repertoire analysis was performed on CSF obtained during their first clinical event. Each CIS patient subsequently developed relapsing-remitting MS within a 6 month period

<sup>B</sup> Single-cell repertoire analysis was performed on CSF obtained from a patient with chronic meningitis. The cause of the disease and the specificity of the rAbs are unknown.

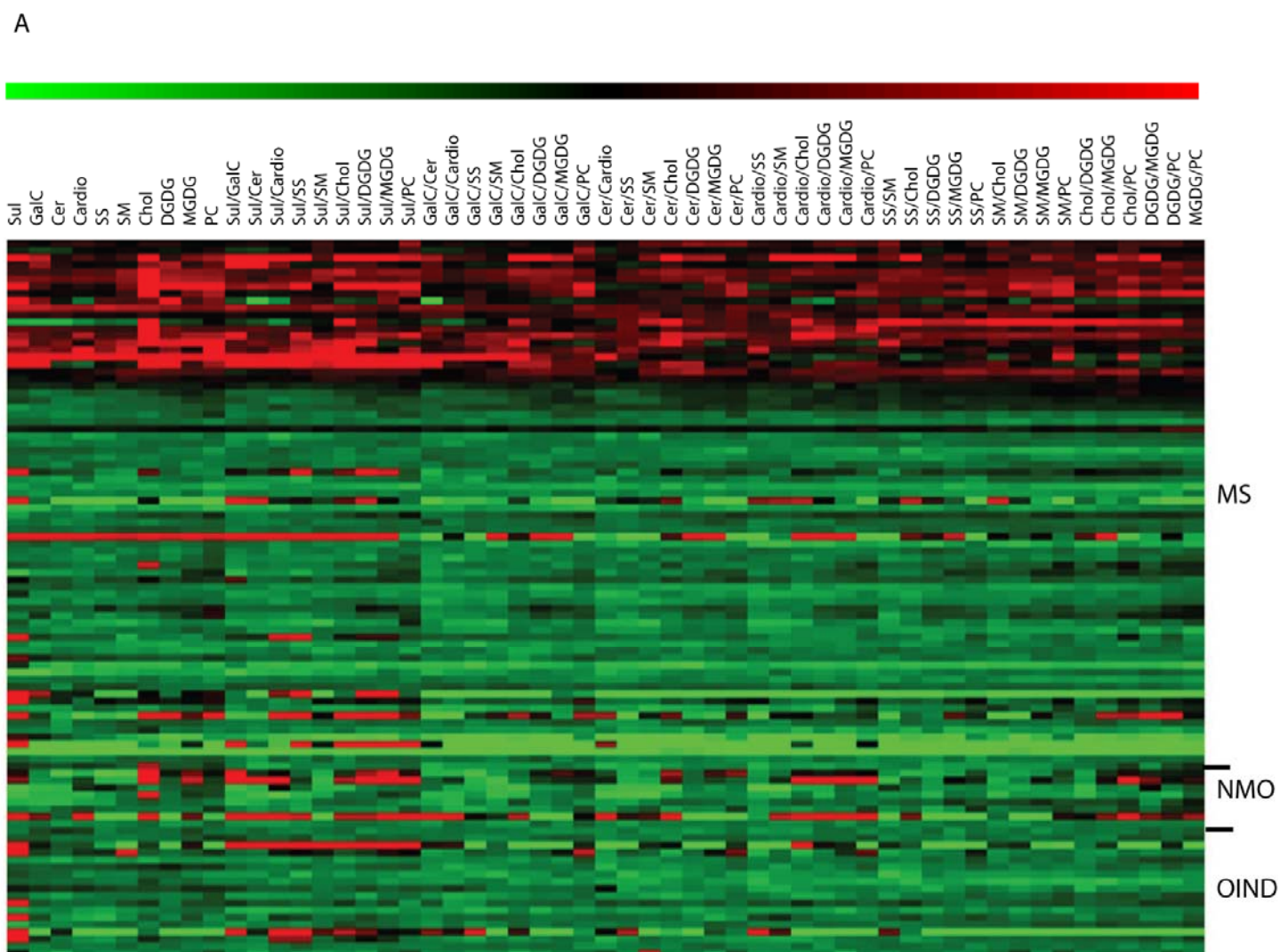
<sup>C</sup> Single-cell repertoire analysis was performed on CSF obtained from a patient with subacute sclerosing panencephalitis (SSPE). All the rAbs have been demonstrated to bind measles virus (MV) proteins (Owens et al 2006b)



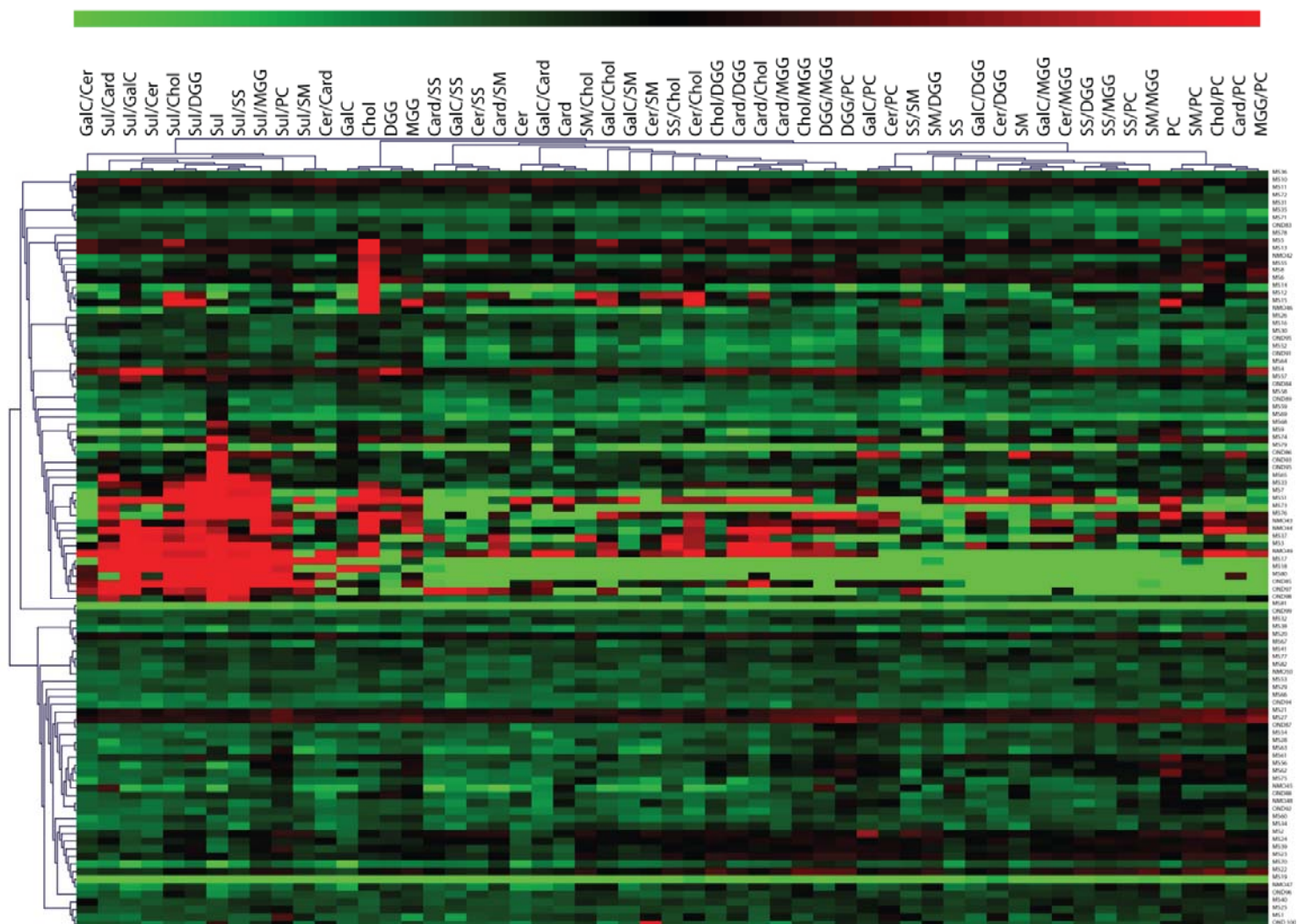
<sup>D</sup> Single-cell repertoire analysis was performed on plasma cells microdissected from SSPE brain tissue by laser capture. Both rAbs bind the MV nucleocapsid protein (Burgoon et al 1999, Burgoon et al 2006)

<sup>E</sup> Single-cell repertoire analysis was performed on CSF obtained during an initial event of optic neuritis. The patient was subsequently diagnosed with neuromyelitis optica. 6 of these rAbs recognize the aquaporin-4 water channel (Bennett et al 2009)

<sup>F</sup> Single-cell repertoire analysis was performed on CSF obtained from a patient with VZV radiculomyelitis. The specificity of the rAbs is unknown.



B

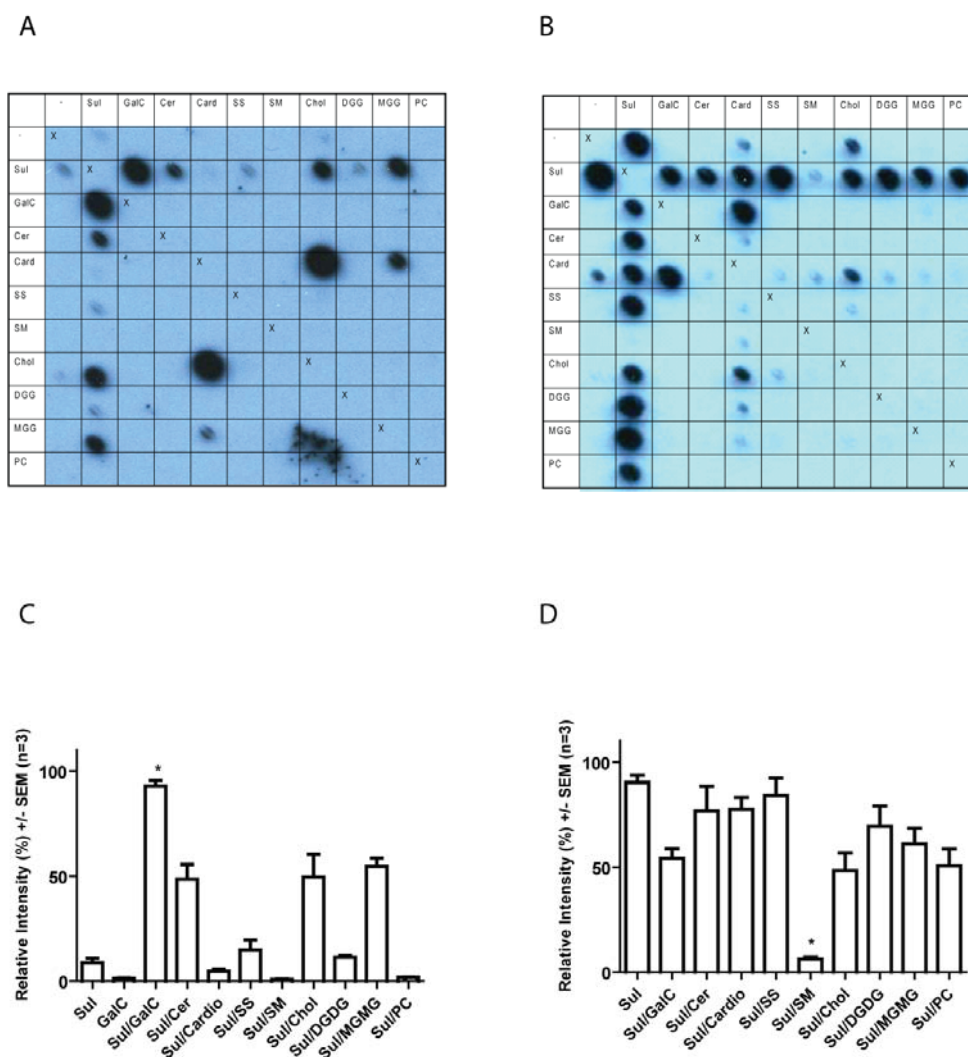


**Figure 5.6. Array analysis for rAbs**

(A) Heatmap depicting reactivity of rAbs derived from MS, NMO and OIND patient CSF, created as before. The lipid antigens are displayed as column headings and each row represents an individual rAb.

(B) Covariance clustering has been performed revealing two populations of lipid reactive antibodies; one directed against sulfatide or sulfatide/lipid complexes and the other against cholesterol. MS rAbs do not segregate with unique binding profiles, as similar reactivities are also seen in OND rAbs (19% vs. 37%).

The array also demonstrated that a single rAb could bind an epitope formed by one lipid pair (Sulf/GalC) and another lipid pair comprising a combination of two structurally unrelated lipids (cholesterol/cardiophilin) (Figure 5.7. and Figure 5.8). This again suggests that antigen shapes could be mimicked by complexes generated from several structurally different lipids, or that certain antibodies are able to bind multiple antigens. The array patterns obtained using individual rAbs reproduced many features observed when arrays were probed with CSF from unrelated MS patients indicating that these anti-lipid antibodies represent commonly represented components of this intrathecal response. Importantly, these specificities were not unique for MS as lipid reactive rAb were also recovered from clonally expanded CSF B cells isolated from OND patients (Figure 5.6 and Figure 5.10). This suggests that MS does not differ from OND with respect to the initial recruitment/expansion of these B cells, but may promote their retention and activation enabling them to make a sustained contribution to the intrathecal antibody response. Interestingly data from our collaborators show (Table 5.3) that several of these lipid-reactive OND rAbs also bind defined protein antigens. This is not a unique phenomenon as demonstrated by the specificity of neutralizing anti-HIV antibodies which can recognize complex epitopes formed by HIV gp41 and phospholipids such as cardiophilin (Alam et al. 2007).

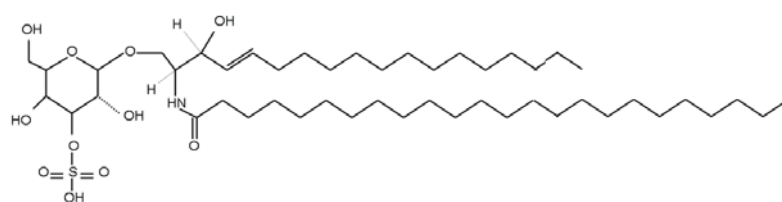


**Figure 5.7. Modulation of rAb binding by lipid partnerships**

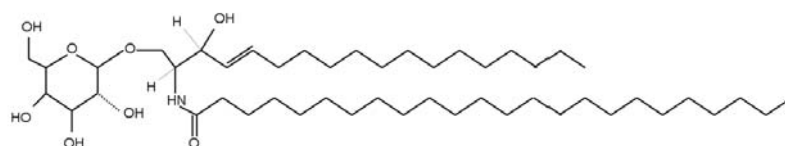
(A, C) Blot and quantification of MS rAb 3 with anti-lipid complex reactivity. Binding to the complex of sulfatide and galactocerebroside is increased by 82.84% ( $p=0.004$  paired t test,  $n=3$ ) compared to the sum of the mean intensities recorded from the individual lipids.

(B, D) Blot and quantification of MS rAb 76 with binding to sulfatide inhibited when sulfatide is complexed with the lipid sphingomyelin ( $p<0.0001$  GLM ANOVA with Tukey,  $n=3$ )

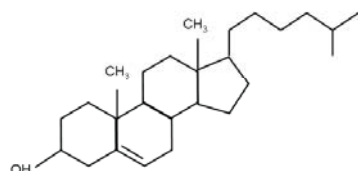
A



B



C



D

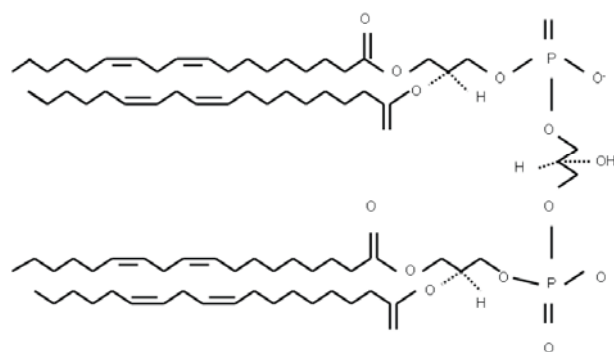
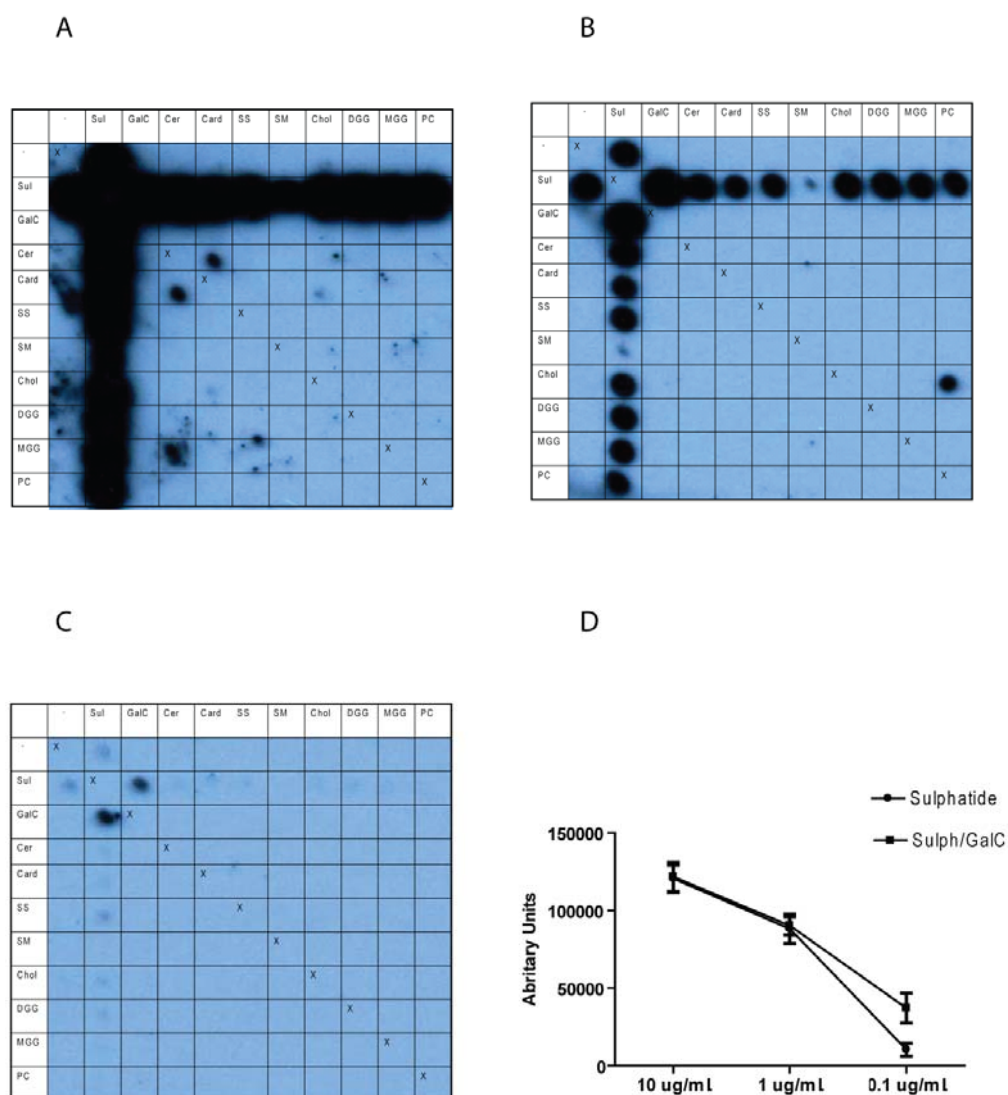


Figure 5.8. Structures of lipid complex partners recognized by MS rAb 3

- A. Sulfatide
- B. Galactocerebroside
- C. Cholesterol
- D. Cardiolipin





**Figure 5.9. Serial dilution of MS rAb 17**

A. Blot of MS rAb 17 10µg/ml

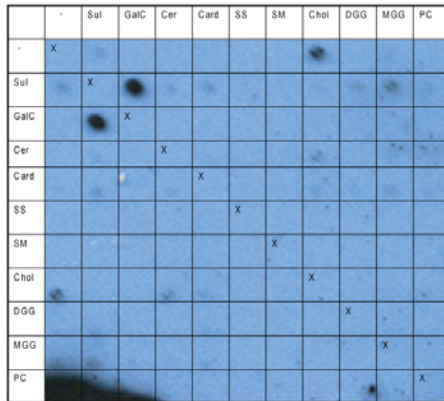
B. Blot of MS rAb 17 1µg/ml

C. Blot of MS rAb 17 0.1µg/ml

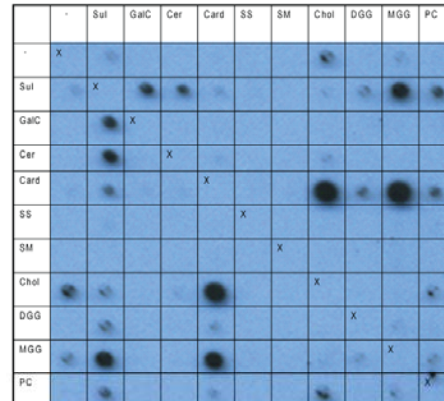
D. Quantification of MS rAb 17 binding to sulfatide and sulfatide/galactocerebroside complex. At a concentration of 0.1µg/ml binding to the complex created by sulfatide and galactocerebroside is enhanced ( $p=0.0143$ , paired t test,  $n=3$ )



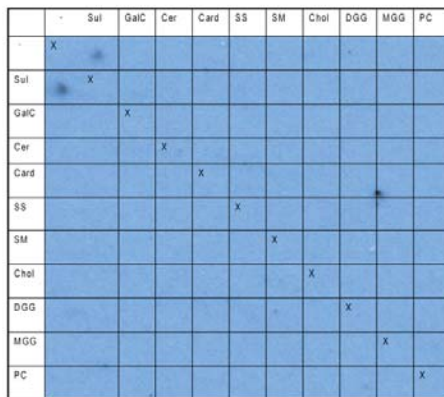
A



B



C



D

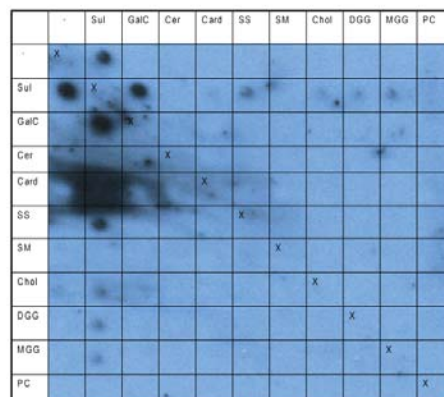


Figure 5.10. Illustrative blots from NMO and OIND patients

A. Blot of NMO rAb 43

B. Blot of NMO rAb 44

C. Blot of OIND rAb 93

D. Blot of OIND rAb 97

### 5.3.3. Direct analysis of individual OCB antibodies

We then collaborated with another group led by Dr K Dornmair (Munich, Germany). They used a novel approach to directly analyze individual OCB that were first separated by high-resolution isoelectric focusing from other OCB and the polyclonal background in the CSF, then analyzed by mass spectrometry, and finally produced as recombinant Fab-fragments. (unpublished data) This method involved the purification of disulfide-linked IgG heavy and light chains of single OCB spots by protein-G chromatography, deglycosylation, IEF and SDS-gel electrophoresis under non reducing conditions throughout. The identified peptide masses were aligned to patient specific IgG transcriptome databases that were generated in parallel from the CSF cells by amplifying, cloning, and sequencing the IgG-H, - $\kappa$  and - $\lambda$  chain transcripts. To identify 5 matching IgG-H and IgG-L chain pairs, they only considered characteristic peptides, i.e. peptides that contained amino acids that were introduced by somatic hypermutations, or comprised the hypervariable complementary determining region 3 (Obermeier et al. 2008). They managed to isolate 3 matching IgG-H and IgG-L chain pairs from patient HM-63, one pair from patient 1039 and from patient NS-52 (Table 5.4).

The lipid reactivity of these HIS<sub>6</sub> tagged OCB-derived Fab-fragments was then investigated using the combinatorial lipid array. In keeping with our previous results, where over 20% rAbs (20/73) bound lipid, one of these five antibodies bound lipid, specifically binding sulfatide and sulfatide lipid complexes (Figure 5.11).

#### 5.3.4. Immunohistological analysis of recombinant antibody binding to primary myelinating cultures

In order to determine whether the lipid binding profiles of rAbs observed in glycoarrays correlate with any binding to equivalent surface antigens in live neural tissues, primary myelinating cultures that comprise a diverse range of specialised neuronal and glial membranes were selected for study. The widely used monoclonal anti-sulfatide antibody O4, which is known to readily bind sulfatide in neural membranes, was used as a comparator for 8 rAbs that were tested. In these studies, none of the rAbs bound surface antigens, in contrast to the intense surface binding seen with the O4 antibody (Figure 5.12. (C) (iv)-(vi)). This indicates that sulfatide-containing complexes recognised by the MS rAb are not accessible within the plane of the normal living membrane, and are therefore unlikely to provide targets for primary antibody-mediated demyelination. The likely explanation for the marked difference between O4 and rAb binding behaviour is shown in Figure 5.12. (A) and (B), in which O4 can be seen to only bind sulfatide when in complex with sphingomyelin, in striking contrast to the typical rAb behaviour, in which sulfatide binding is inhibited by sphingomyelin.

Table 5.4. Clinical data of the three MS patients from whom individual OCBs were analyzed.

Patient	[IgG] <sup>CSF</sup> (g / l)	IgG <sup>q</sup>	total cells (cells / ml)	B-cells (B-cells / ml)	disease duration (years)
NS-52:	0.057	4.9	7,000	350	8
HM-63	0.146	14.7	40,700	2,035	2
1039	0.051	5.5	3,000	150	5

We list the IgG concentrations in CSF, [IgG]<sup>CSF</sup>, the IgG quotient  $\text{IgG}^q = [\text{IgG}]^{\text{CSF}} \times 10^3 / [\text{IgG}]^{\text{serum}}$  as quotient of the IgG concentrations in CSF and serum, and the number of cells of the B-lineage No. <sup>B-cells</sup> calculated as 5 % of the total cell number from CSF (Meinl et al. 2006).

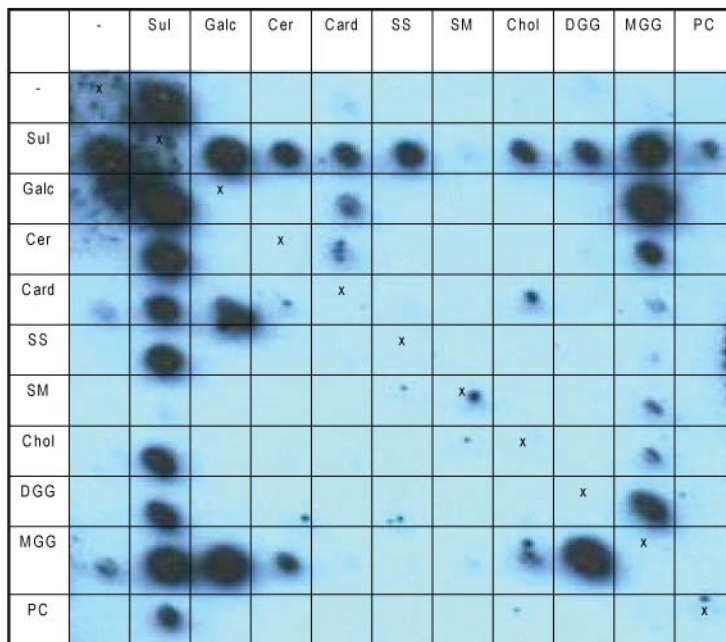
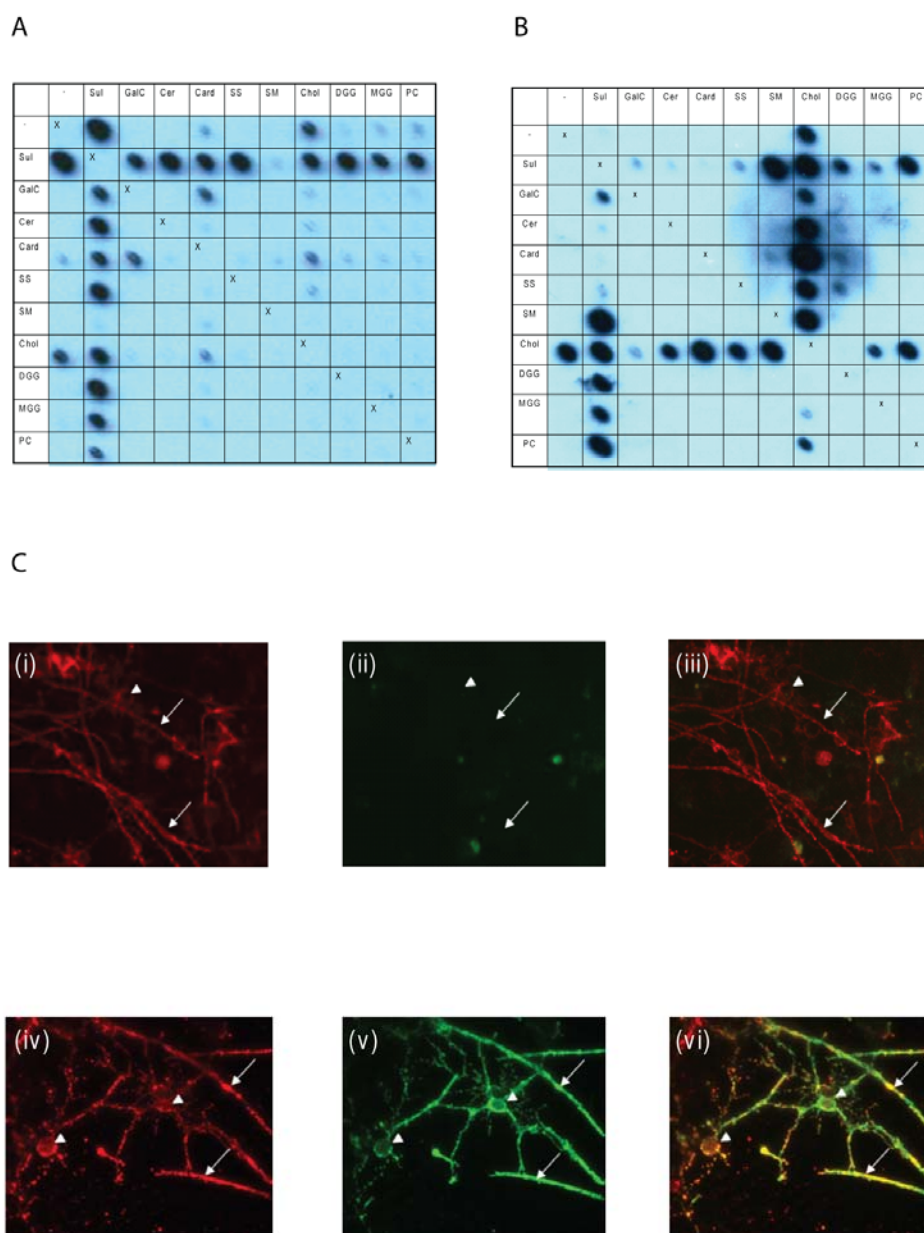


Figure 5.11. Lipid reactivity of Fab-HM-63-s9

Illustrative blot of lipid reactivity of lipid reactive Fab



**Figure 5.12 Lipid reactivity of rAb and O4 antibodies**

A. Blot of MS rAb 76

B. Blot of mAb O4

C. Unfixed myelinating cultures derived from embryonic rat spinal cord were stained at 4°C with either (i)-(iii) MS derived rAb 76 (10µg/mL) or (iv)-(vi) mAb O4 (10µg/ mL) to visualize sulfatide (green) followed by the MOG-specific mAb Z2 (10 µg/mL) to identify oligodendrocytes and myelin sheaths (red). Control cultures were stained with polyclonal human IgG, mouse myeloma proteins and/or secondary antibody alone. In no case did immunoreactivity for MOG (red) at the surface of oligodendrocytes (arrow head) or myelinated internodes (small arrows) co-localize with bound human rAb ((ii), green) as

demonstrated in the merged image (iii). Staining obtained using rAb was restricted to a diffuse background indistinguishable from that observed when cultures were stained using polyclonal human IgG pooled from multiple donors. In contrast the sulfatide reactive mAb O4 binds co-localizes with MOG on the surface of oligodendrocytes and myelin sheaths (iv)-(vi).

## 5.4. Discussion

The specificity of the intrathecal antibody response associated with MS has been the subject of intense speculation since it was first described over fifty years ago. Collaborating with our colleagues in America and Germany has allowed us to explore the specificities of intrathecally produced antibodies.

The monoclonal agents used in this study were derived from geographically distinct patient populations using two different approaches. Yet both identified lipids as a major target specificity; 27% recombinant antibodies derived from single clonally expanded CSF B cells (20/73) and 1/5 of Fab fragments defining individual OCB exhibiting lipid specificities. Thus two complementary but different approaches identify the role of lipids as targets of the intrathecal B cell response associated with MS, a concept supported by data obtained from analysis of antibody specificities in whole CSF in both this and previous studies(Ilyas et al. 2003)(Kanter et al. 2006).

It is intriguing to note that 4 recombinant antibodies demonstrated anti-lipid complex reactivity to the complex created by sulfatide and galactocerebroside (complex reactivity is again defined using a modified version of Kaida's definition with intensity of IgG directed against the complex being greater than the sum of the intensity of IgG directed against individual lipid partners)(Kaida et al. 2007) . A further 5<sup>th</sup> monoclonal antibody appears to have anti-galactocerebroside/sulfatide complex specificity as lowering the rAb concentration reveals the preferred target is provided by the sulfatide/galactocerebroside complex (see Figure 5.11). These two lipids are structurally very similar, galactocerebroside being one of the simplest glycolipid structures consisting of a galactose molecule

linked to a ceramide. Sulfatide is a galactocerebroside molecule in which the 3'OH moiety on galactose is sulfated(Norton 1977).

These two major glycosphingolipids of central nervous system myelin are reported to interact with one another by *trans* carbohydrate-carbohydrate interactions(Boggs et al. 2010). These lipids face each other in opposed extracellular surfaces of the multi-layered myelin sheath. Dendrimers or silica nanoparticles conjugated with saccharides have been used to mimic *trans* interactions between such cell surface glycolipids. It has been suggested, through the use of such nanoparticles, that galactocerebroside and sulfatide partake in a “glycosynapse”(Boggs et al. 2010). The interaction between the dendrimers and membrane bound lipids resulted in loss of the cytoskeleton and clustering of membrane domains in oligodendrocyte culture systems. The authors suggest that this particular glycosynapse may be important for myelination and/or myelin function. Our data may suggest that this “glycosynapse” may be an antigenic target in Multiple Sclerosis.

The currently described array only uses 10 lipids in heteromeric complexes and does not take into account possible effects due to differences in alpha-hydroxylation, acyl chain length or degree of saturation of the fatty acid. Nevertheless we have identified specificities associated with over 20% of the intrathecal response. We anticipate that increasing array complexity through addition of further lipids and lipid- or membrane- associated proteins will identify additional target antigens.

The role of such antibodies remains as yet undetermined. There is no firm data supporting a role for any known intrathecal antibody in mediating



demyelination as a primary event. However, for sulfatide, produced by oligodendrocytes and highly enriched in myelin and at a critical point during oligodendrocyte differentiation, a pathogenic role of antibody has been suggested (Bansal et al. 1989). The monoclonal antibody O4 that is specific for sulfatide, exacerbates demyelination in animals with experimental autoimmune encephalitis (EAE) (Kanter et al. 2006). This represents a dichotomy of evidence and suggests that the sulfatide binding properties of antibodies may be diverse. To address this we compared the ability of O4 and a selection of CSF derived, sulfatide reactive rAbs, to bind to the surface of myelinating oligodendrocytes in vitro. rAbs do not reproduce the staining pattern of O4; therefore the lipid complexes required for binding cannot be available on the intact live membrane. Intriguingly the mAb O4 binds to the complex formed between sulfatide and sphingomyelin, whereas in the majority of our MS patients and rAbs binding to sulfatide was inhibited when in complex with sphingomyelin. This may suggest that myelin may require to be damaged to expose the necessary and specific lipid antigen binding sites for the recombinant antibodies to bind (Figure 5.13).

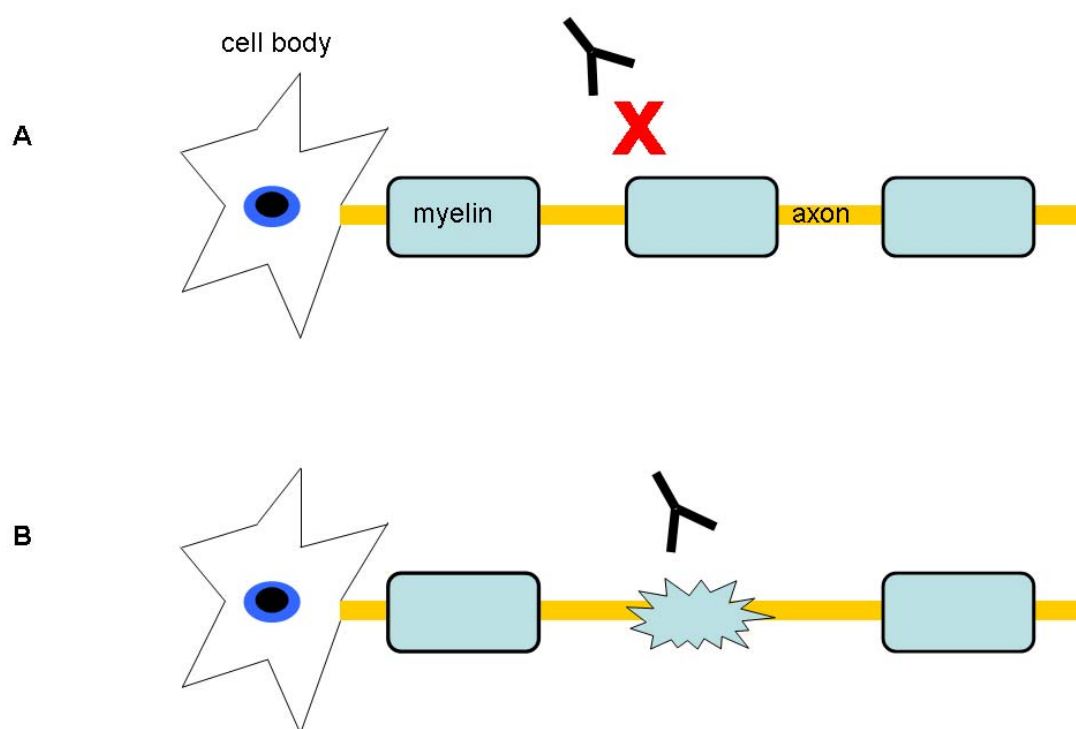


Figure 5.13 Proposed mechanism of binding of MS derived rAbs

- A. Lipid reactive MS derived rAb do not bind antigen when myelin intact
- B. Lipid reactive MS derived rAb may bind lipid complexes when myelin membrane is disrupted and damaged in demyelination

We cannot assume that the population of lipid reactive antibodies present in MS CSF will behave like the mAb O4, in terms of pathogenic activity. Indeed one hypothesis may be that sulfatide reactive intrathecal antibodies may be neuroprotective, for example by enhancing the clearance of myelin debris. Whilst their biological significance still currently remains unclear, identification of their specificity provides powerful avenues towards investigating this experimentally.

In order to further investigate the binding capabilities of these MS derived rAb further studies are planned. Both rat and mouse (where a higher level of myelination compared to the rat system is commonly observed) derived myelinating culture systems will be utilised to investigate this.

Demyelinating injury to these culture systems could also be introduced though the addition of anti-MOG (myelin oligodendrocyte glycoprotein) antibody with a source of complement. My hypothesis is that binding of these rAbs would only occur when demyelination has occurred releasing lipid complex antigens which the rAbs then recognize.

Of course we have only screened an array of 10 lipids and their one to one combinations. It is known that there is variability in the degree of hydroxylation, length of fatty acid chains and degree of esterification within myelin based lipids(Norton 1977). This adds another level of complexity to our continued search for target antigens. In addition it is known that lipids reside in membrane microdomains with proteins as well as other lipids. The possibility that antigenic targets consisting of several lipids and proteins exists, and deserves further exploration. This would not be feasible on the current template which is limited in the number of antigens which can be spotted therefore the next step is to create a true microarray of lipids where these questions may be addressed.

## Chapter 6. Development of lipid microarray

### 6.1. Introduction

The combinatorial array is clearly advantageous in the search for novel lipid complex antigens however it has many limits. To date I have simply investigated antibody responses to single lipids and pairs of lipids. An additional level of complexity would be investigating responses against groups of three or more lipids. In addition it is known that there is variability in the degree of hydroxylation, length of fatty acid chains and degree of esterification within myelin based lipids (Norton 1977). This adds further levels of complexity to our continued search for target antigens. Furthermore lipids reside in membrane microdomains with proteins as well as other lipids (Simons et al. 1997). The possibility that antigenic targets, consisting of several lipids and proteins, exists and deserves further exploration. This would not be feasible on the current template which is limited in the number of antigens which can be printed. To help address these issues the next step is to create a “true microarray” of lipids which would allow a greater number of lipid antigens to be applied per slide. This would allow us to multiplex the assays, by printing very small spots of libraries of multiple lipids onto a small area of substrate, which can then be interrogated with small amounts of samples.

Despite the technical issues I encountered with the fluorescent technique (see Chapter 2; 2.2.5.) I next investigated creating a microarray method. These experiments were carried out with Dr Susan Gannon of the RASOR (Radical Solutions for Researching the Proteome) consortium of the University of Glasgow. Fluorescent based microarrays can offer advantages where sensitivity of analyte detection is enhanced. Again I used the same experimental outline, where lipid is printed onto a membrane, which would then be incubated with the serum/antibody of interest. A secondary antibody which has been labelled with a fluorescent dye would then be used

to visualize any reactivity (protocol outlined in Figure 6.1.). A non-contact arrayer (see Figure 6.2.), Perkin Elmer Piezorray<sup>TM</sup> was used to deposit 1nl volumes (in the form of 3 droplets of 330pl each) of 100ug/ml gangliosides prepared in methanol on slides covered with PVDF membranes.

Each individual slide is composed of 16 printed subarrays. Each subarray was positioned on a 10 x 10 grid with 10 replicates of each capture spot composition. Spacing between the spots was set at 500µm and individual spot diameter was ~300nm. 16 subarrays were replicated per individual microscope slide. After printing, slides were allowed to dry overnight. Slides were then inserted into the 16 well FASTframe (Whatman, UK) for blocking and probing. The application of this frame allows each individual array to be potentially probed by 16 specific antibodies. Post block array wells were rinsed with PBS and primary antibody/patient serum was then applied (100µl for 1 hour). A PBS wash followed, then secondary antibody was added (80 µl for 30 minutes). The secondary antibody used was either anti-human/mouse IgG labelled with the fluorescent AlexaFluor dye 647. A final PBS was followed and slides were allowed to dry before scanning.

Probed arrays were imaged on the ScanArray Express at excitation wavelength 633nm using an AlexaFluor 647 built in filter (at laser power 90 and PMT 40) at the resolution of 10 µm. Quantitative function within the ScanArray Express software was used to convert the fluorescence levels in the array images into relative fluorescence units. The individual spot signal used is the mean spot fluorescence minus background.

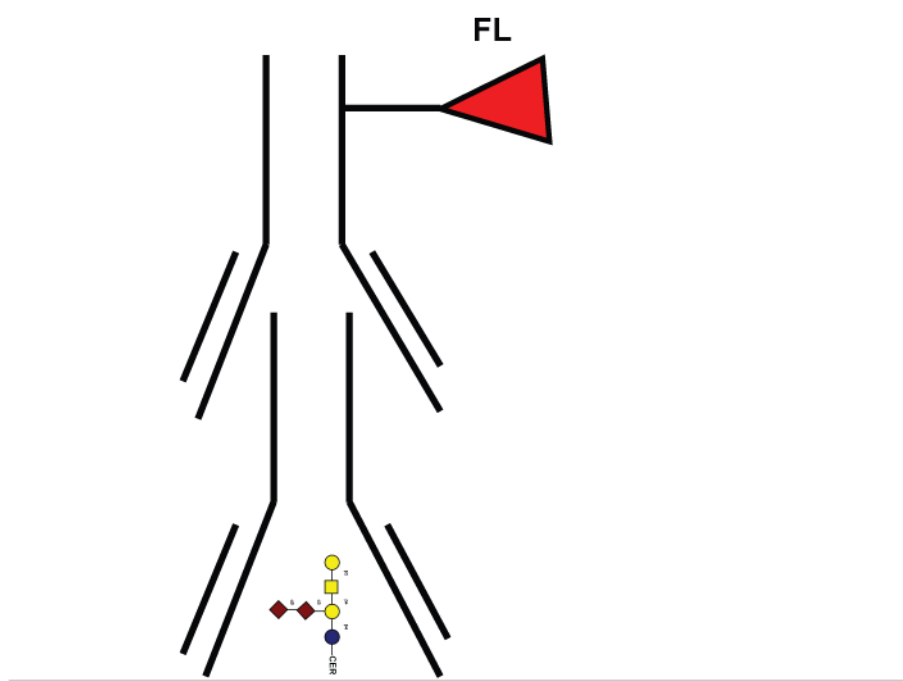
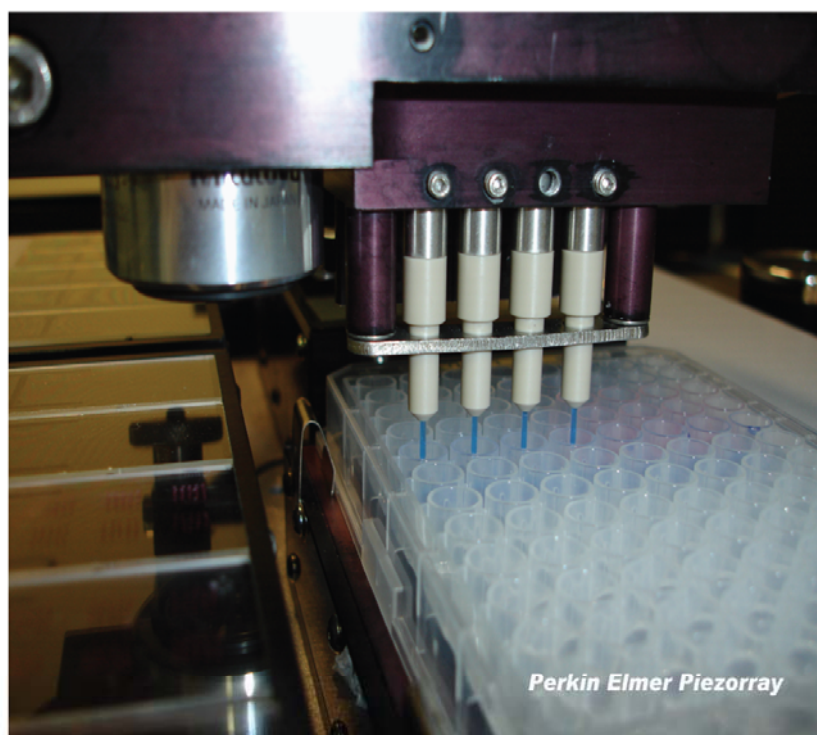


Figure 6.1. Protocol of microarray experiment

Lipid antigen (GD1b illustrated here) adheres to hydrophobic membrane exposing carbohydrate moiety. Primary antibody e.g. human GBS serum binds to lipid. Secondary anti- human IgG (which is labelled with a fluorescent dye) binds to the primary antibody. The fluorescent intensity is then measured.

A



B



C

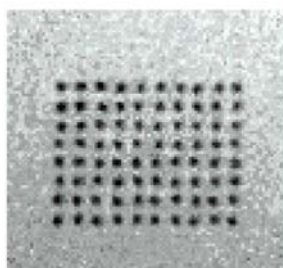


Figure 6.2. Microarray platform

A. Perkin Elmer Piezoarray<sup>TM</sup>

B. Whatman FAST frame

C. Black and white image of an individual array. Naphthol Blue Black (Sigma-Aldrich, UK) has been added to the ganglioside spotting solution at a concentration of 0.1mg/ml to assess the quality of spotting.

## 6.2. Development of microarray protocol

### 6.2.1. Slide platform

Initial experiments were carried out using homemade slides. PVDF membranes were cut (to the size of a glass microscope slide) and affixed using UHU glue (Ryman, UK) to plain glass microscope slides (VWR International, UK). A number of PVDF membrane types were used including 0.2um pore size PVDF (Invitrogen, UK) and 0.45 pore size Immobilon FL PVDF (Sigma-Aldrich, UK) (see Figure 6.3). Each slide was placed into a slide holder which divided the slide into 16 separate chambers. Within each pad (labelled 1-16) 10 rows of lipid were spotted. GM1 was the only lipid printed here. Each individual subarray is composed of 10x10 GM1 spots. Each subarray was then probed with the mouse derived monoclonal anti-GM1 antibody (IgG), DG2. AlexaFlour 647 labelled goat anti-mouse IgG (Invitrogen) was then used as a secondary antibody.

Both slides were associated with a large amount of background artefact and inconsistent spotting of the lipid. This inconsistency of spotting could be secondary to the fact that we were using lipids dissolved in the solvent methanol. It is noteworthy that this arrayer is primarily used to spot proteins dissolved in either water or PBS.



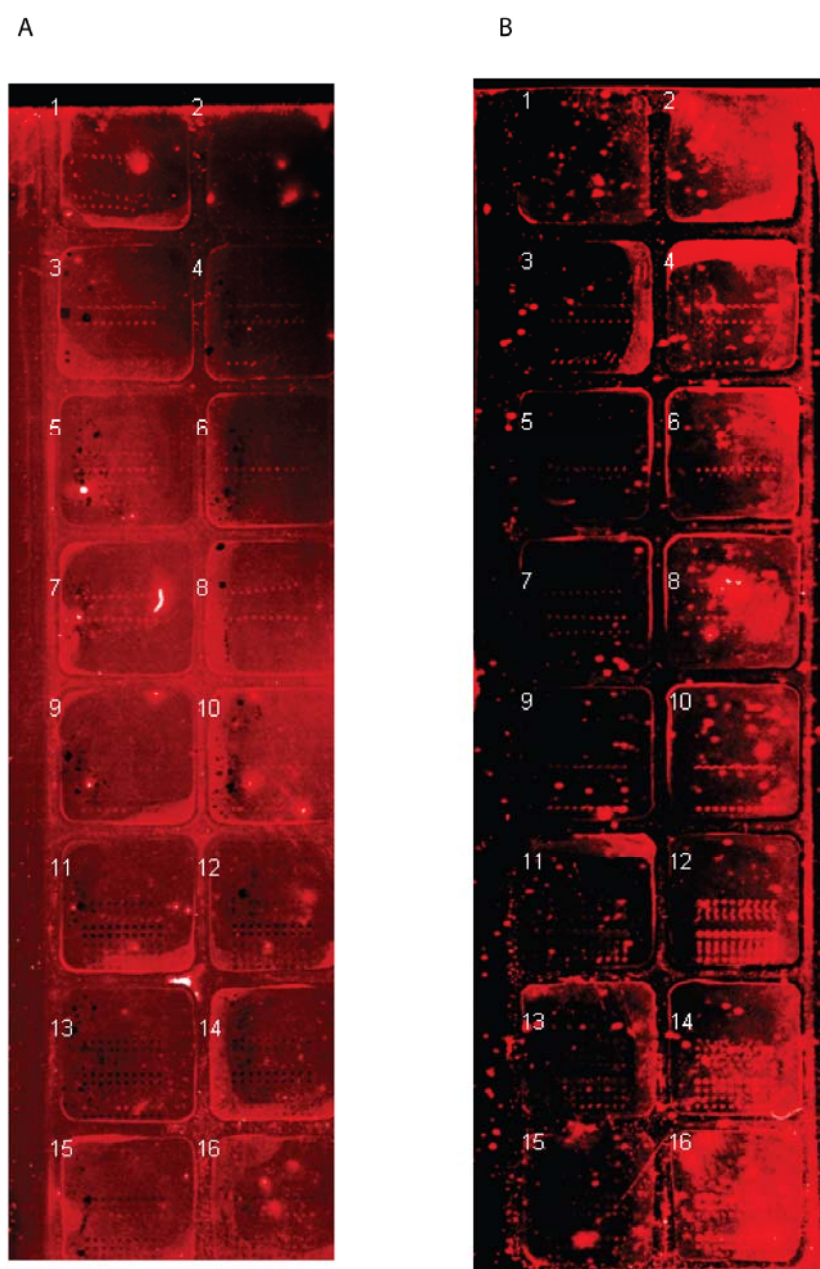


Figure 6.3. "Homemade" slides

The numbers on the slide relate to each individual subarray. Here only the lipid GM1 was spotted and all subarrays were probed with the anti-GM1 antibody DG2.

A. Slide covered with PVDF membrane (Invitrogen, UK)

B. Slide covered with PVDF "Immobilon FL" membrane (Sigma-Aldrich, UK)

### 6.2.2. Investigation of blocking agents and addition of water and dyes to lipid preparations

As there are a large number of sub arrays per slide I used individual experiments to address multiple issues. I next investigated 3 issues with one experiment. I investigated different methods of blocking (see Figure 6.4.) using 2% BSA, 2% milk and no blocking step. Spot quality with milk was poor with additional background artefact (Figure 6.4. (B)). When the blocking step was omitted the signal from individual spots was saturated as indicated by the spots staining white in Figure 6.4. (C).

During this experiment I also investigated using lipid antigen dissolved in different concentrations of methanol and water. In addition I began to look at the effect of adding a visible dye to the lipid mix. An advantage of adding dye at this stage would be that, after a print run, each subarray could visually inspected for spot quality. However there continued to be considerable variation in spot size and intensity that was unpredictable (see Figure 6.5.). Despite these limitations, I was able to ascertain that lipid dissolved in a mixture of methanol and water could serve as a target antigen (see Figure 6.6.). The addition of a dye to the mix did not appear to significantly affect printing of lipid which continued to be unpredictable. However both xylene cyanol blue (Grisp research solutions, Portugal) dye and chromatrope 2R (Sigma-Aldrich, UK) provided quite light staining of the PVDF membrane which was difficult to view with the naked eye.

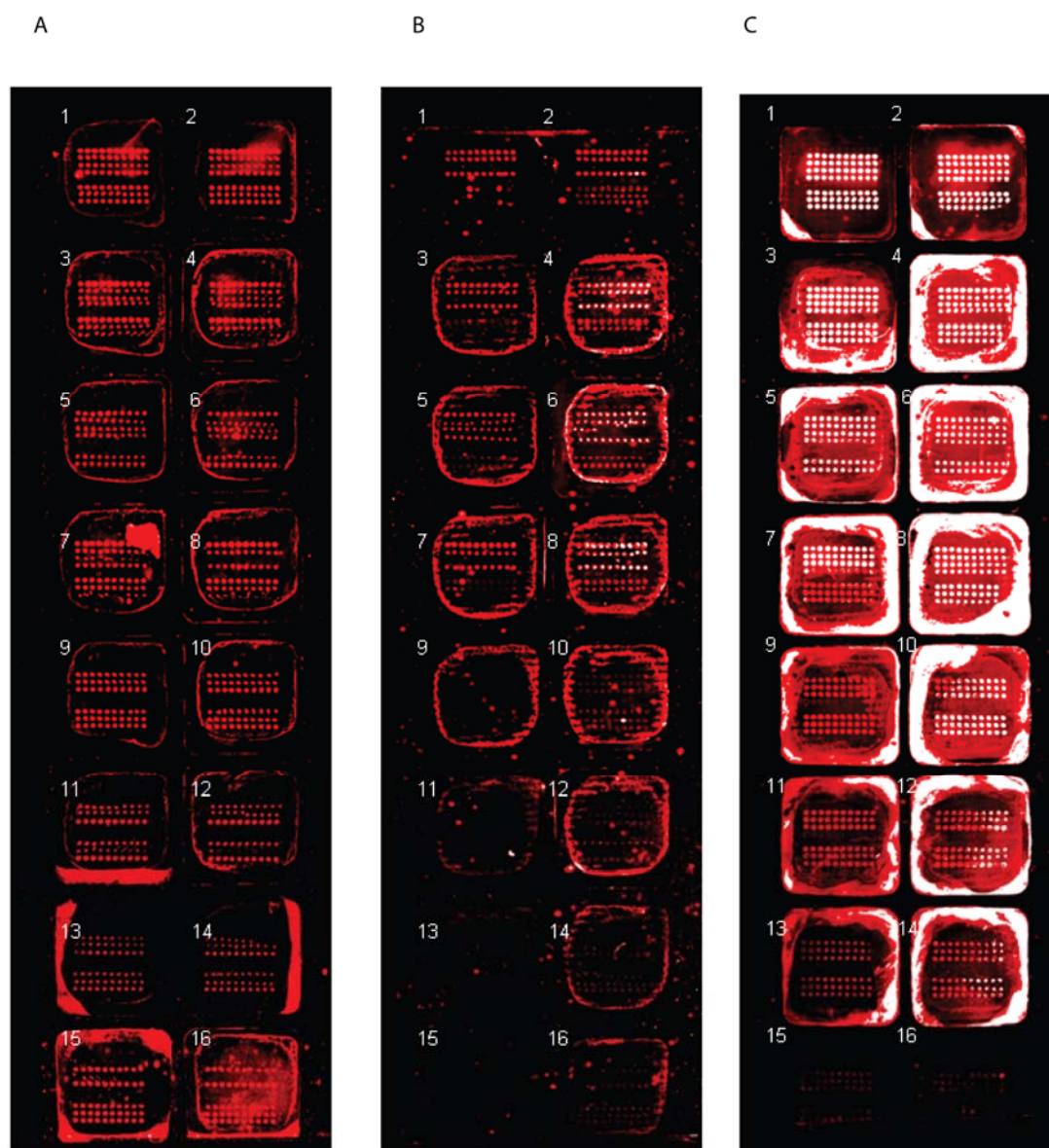


Figure 6.4. Different methods of blocking

A. 2% BSA (FAF) in PBS

B. 2% milk

C. No block

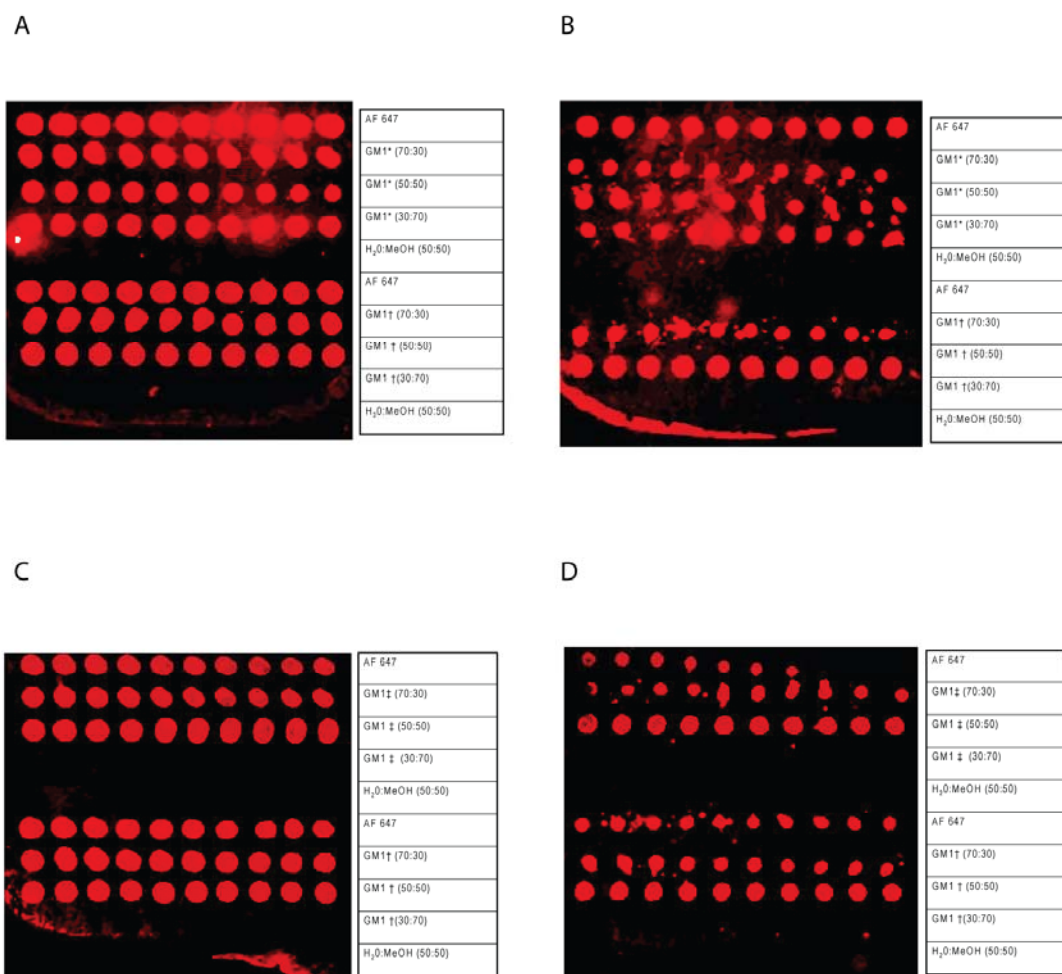


Figure 6.5. Illustration of individual subarrays from the BSA blocked slide (Figure 6.4. (A)). Lipids have been printed using a range of methanol: water concentrations and in a variety of dyes. For individual templates see (A)-(D). All subarrays were probed with the anti-GM1 mouse monoclonal antibody DG2.

Key:

\* xylene cyanol blue dye added to lipid mix

‡ chromatrope 2R dye added to lipid mix

† no dye added to lipid mix

A. Subarray 1; GM1 spotted with (xylene cyanol blue) and without dye

B. Subarray 6; GM1 spotted with (xylene cyanol blue) and without dye

C. Subarray 9; GM1 spotted with (chromatrope 2R) and without dye

D. Subarray 14; GM1 spotted with (chromatrope 2R) and without dye

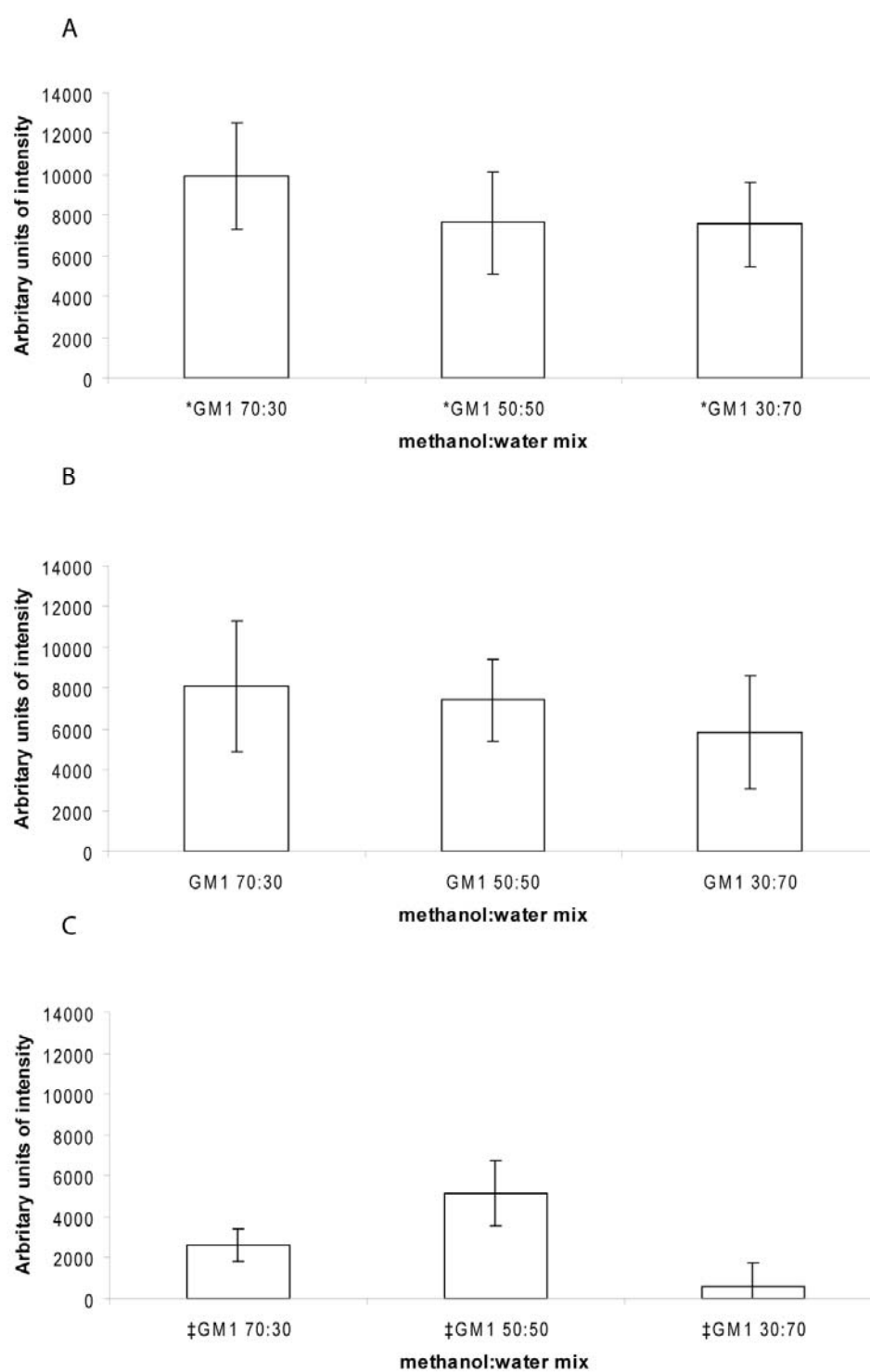


Figure 6.6. Effect of addition of water to lipid:methanol solution

A. GM1 with xylene cyanol blue

B. GM1 alone

C. GM1 with chromatrope 2R

### 6.2.3. Preparation of lipids with dye

From now on all lipid used was dissolved in a mixture of methanol and water (70:30) unless otherwise stated. I also used new PVDF covered slides called “Superprotein” slides (ArrayIt, USA) in this experiment.

I printed the same template across 16 subarrays per slide (see Figure 6.7.). This template included GM1 and GD1b (for template details see Figure 6.7 (C), (D)). The lipids were printed alone and also in combination with amido black dye. Amido black (Sigma-Aldrich, UK) is an amino acid (molecular structure shown in Figure 6.8. (D))staining diazo dye which readily dissolves in both methanol and water. Staining of individual spots on the PVDF membrane was easily visible to the naked eye.

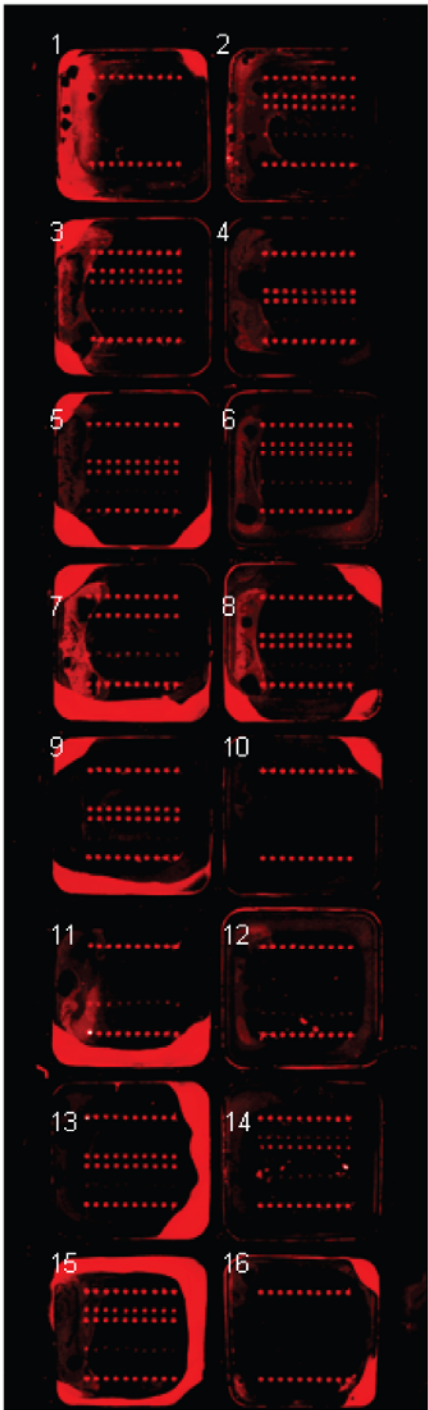
It is clear from Figure 6.7. that there was considerable variation in the spot intensity achieved. Even for the inherent positive control, Alexa Fleur 647 (AF 647) which is dissolved in 0.1% BSA in PBS, the coefficient of variation is high at 15.74%. Investigating the 8 subarrays (2,3,6,7,10,11,14 &15 in Figure 6.7. (A)) in which DG2 was probed leads us to a coefficient of variation of 73.92%. It is worth remembering though the slides are printed using two programs, with the first programme printing the first 8 (labelled 1-8) subarrays and the second printing the next 8 (labelled 9-16) subarrays. If we calculate the coefficient of variation for GM1 for the first programme (i.e. from subarrays 2, 3, 6, & 7, which is 18.49%) then coefficient of variation approaches that seen for AF 647.

In this experiment I used two concentrations of lipids dissolved in dye, 100µg/ml and 10µg/ml. The lower lipid concentration was too low to reliably detect antibody as illustrated in Figure 6.8. (B). Data here has been extracted from subarray 6 (Figure 6.8.(A)) in which DG2 binds to GM1 spots. There is a significant reduction in fluorescent intensity in the GM1 spots created by the lesser concentration of dye combined with GM1. However there is no difference in between the GM1 with and without dye. In fact across 8 subarrays there was no statistically significant difference in binding between the GM1 with and without dye.

However further work investigating the concentration curves of monoclonal antibody binding suggested that, in particular cases, there was an attenuation of antibody binding to lipid when prepared with dye (see Figure 6.9 (A)).



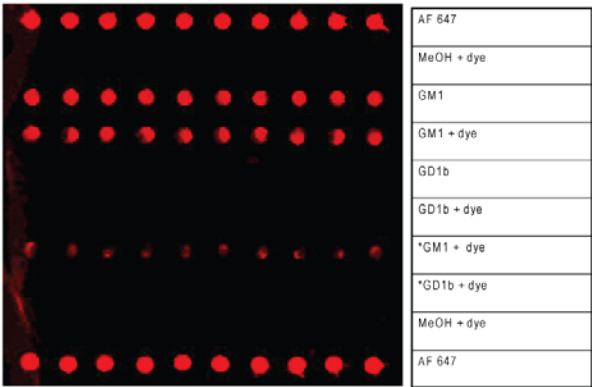
A



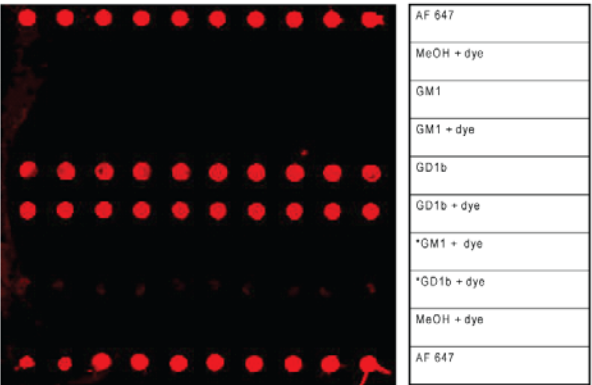
B

1	Blank
2	DG2
3	DG2
4	MOG3
5	MOG3
6	DG2
7	DG2
8	MOG3
9	MOG3
10	DG2
11	DG2
12	MOG3
13	MOG3
14	DG2
15	DG2
16	Blank

C



D





**Figure 6.7. Printing of lipid with amido black dye**

The dye 0.2mg/ml amido black has been used at a concentration of 0.2mg/ml. All lipids are printed in a concentration of 100µg/ml unless otherwise stated.

\* lipids here are printed in a concentration of 10 µg/ml

A. Illustrative image of whole slide

B. Key of antibodies used to probe individual subarrays. DG2 is monospecific for GM1 and MOG3 is monospecific for GD1b. Arrays 1 & 16 were probed with PBS only.

C. Illustrative subarray 6 demonstrating binding of DG2 to GM1

D. Illustrative subarray 5 demonstrating binding of MOG3 to GD1b

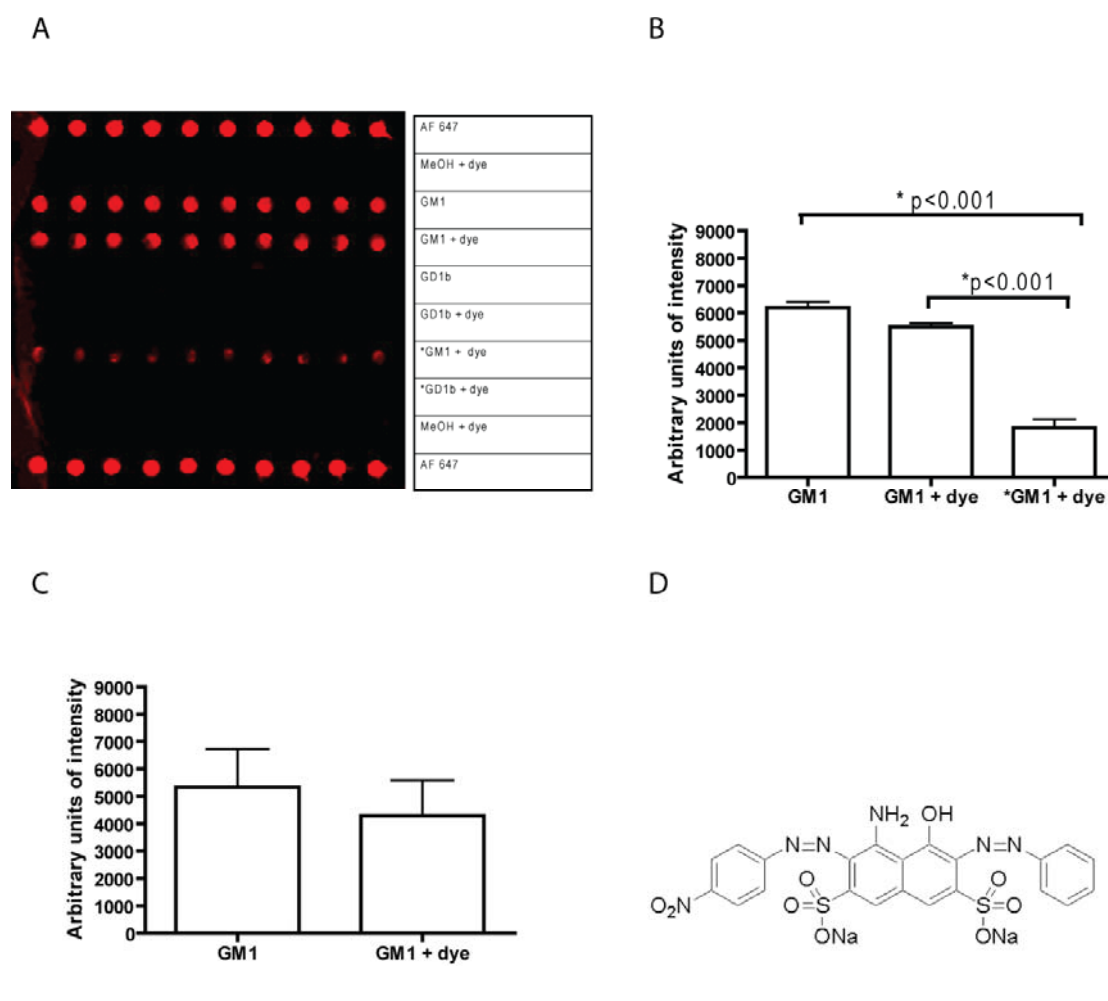


Figure 6.8. The effect of dye (amido black) on binding of antibody

\* lipids here are printed in a concentration of 10  $\mu\text{g/ml}$

A. Illustrative image of subarray 6

B. Bargraph illustrating the binding of DG2 to subarray 6. p values quoted are obtained with ANOVA with Tukey

C. Bargraph illustrating binding of DG2 to all 8 subarrays

D. Molecular structure of amido black

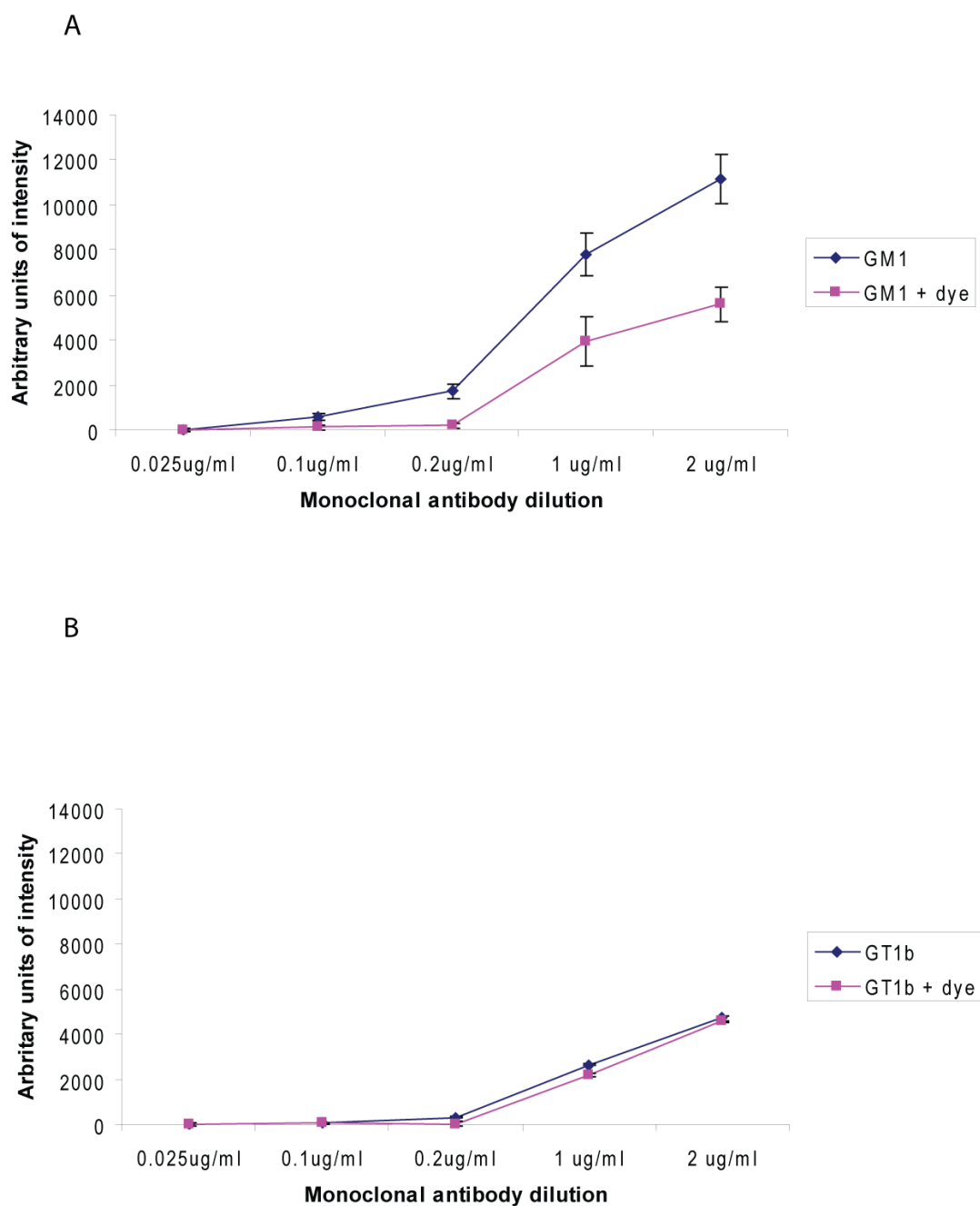


Figure 6.9. Concentration curves for binding of the monoclonal antibodies to lipids prepared with and without dye (amido black).

DG2 binds GM1 preferentially and EG4 binds GD1b and GT1b.

A. DG2 binds preferentially to lipid without dye.

B. EG4 binds binds similarly to lipid with and without dye

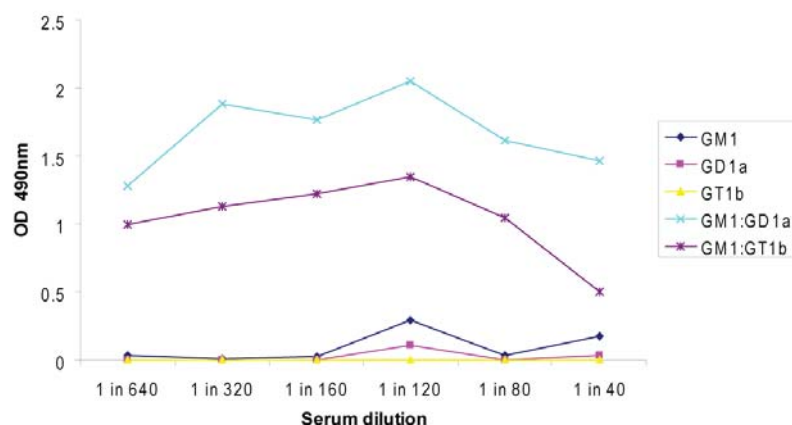
### 6.2.3 Printing of lipid complexes

The next step was to create complexes of lipids using this platform. An option here is to simply print premixed solutions of lipids onto the slide. However this would lengthen the printing time considerably when large numbers of complexes are being investigated. Another option is to “overspot” lipid partners i.e. print single lipids sequentially over the same spatially addressable spot on the membrane thereby creating a complex. I investigated this using serum from a patient with GBS who demonstrated high titre anti-ganglioside complex antibodies using both ELISA and combinatorial blot methods (see Figure 6.10). Using both techniques high titres of IgG is observed to two lipid complexes; GM1:GD1a, and GM1:GT1b.

For this experiment I used goat anti-human IgG labelled with Alexa Fluor 647 (Invitrogen, UK) as a secondary antibody. Figure 6.11. illustrates individual subarrays. Another patient, D.B. with known high anti-GM1 titres of >12500 on ELISA, was used as a positive control sera to demonstrate binding to GM1. Higher levels of binding were observed for the complexes created by GM1:GD1a compared to either single lipid isolation using both methods of placing the lipid complexes (overspot method  $p = 0.0443$ , paired t test,  $n=4$ , and premixed lipid  $p = 0.0233$ , paired t test  $n=4$ ). Binding to the complex created by GM1:GT1b was not as strong. Using the overspot method binding was preferential for the complex ( $p = 0.0344$ , paired t test,  $n=4$ ) however this was not observed using the premixed samples.

All assays in this experiment were carried out with and without the addition of amido black dye (see Figures 6.12 & 6.13.). Whilst the addition of dye does not have a significantly attenuating effect on lipid binding there is considerable variation observed across the concentration curves when dye is added to the lipid.

A



B

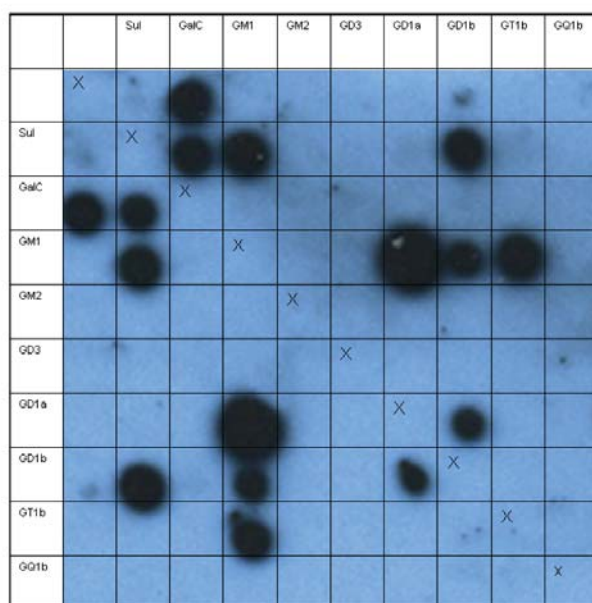
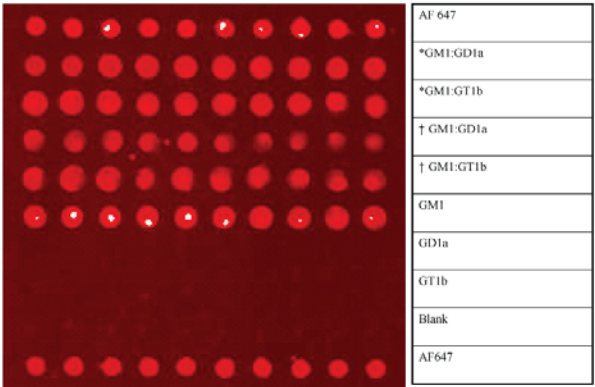


Figure 6.10. ELISA and combinatorial blot of serum from a GBS patient, P.J.

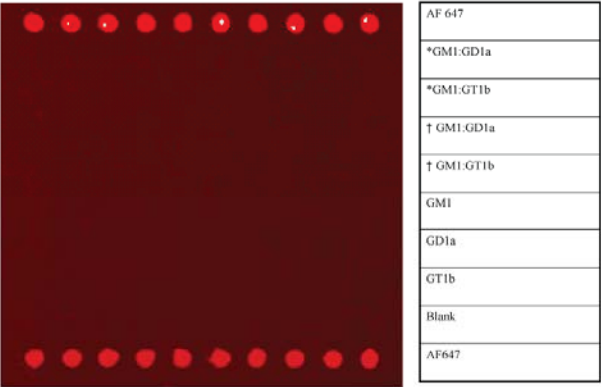
A. ELISA demonstrating preferential binding to the complexes created by GM1: GD1a and GM1: GT1b

B. Illustrative blot demonstrating preferential binding to the complexes created by GM1:GD1a and GM1:GT1b. Row and column headings reveal the complex at each location. "X"s represent the negative controls (methanol) which act as a line of symmetry for duplicate spots within the same membrane.

A



B



C

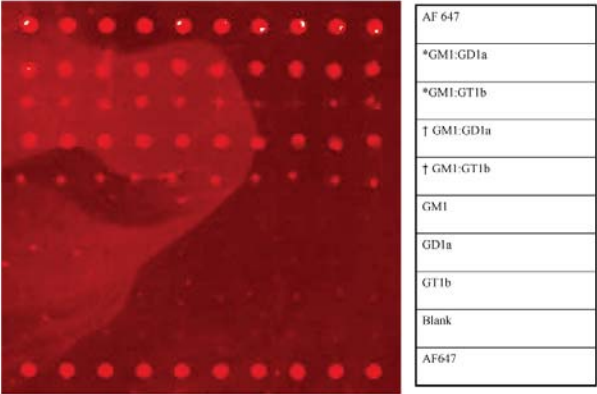


Figure 6.11. Illustrative subarrays demonstrating binding of antibody to single lipids and complexes of lipids.

Templates are outlined alongside each subarray.

A. Subarray demonstrating binding of serum from a GBS patient D.B.. Here IgG binds to GM1 in a complex independent manner i.e. binding occurs to GM1 alone and when complexed with other lipids.

B. Subarray demonstrating no binding to lipids. This represents serum from a healthy control.

C. Subarray here demonstrates binding of serum from another GBS patient P.J. (the same patient in Figure 6.7.). Here binding occurs preferentially to complexes of lipids

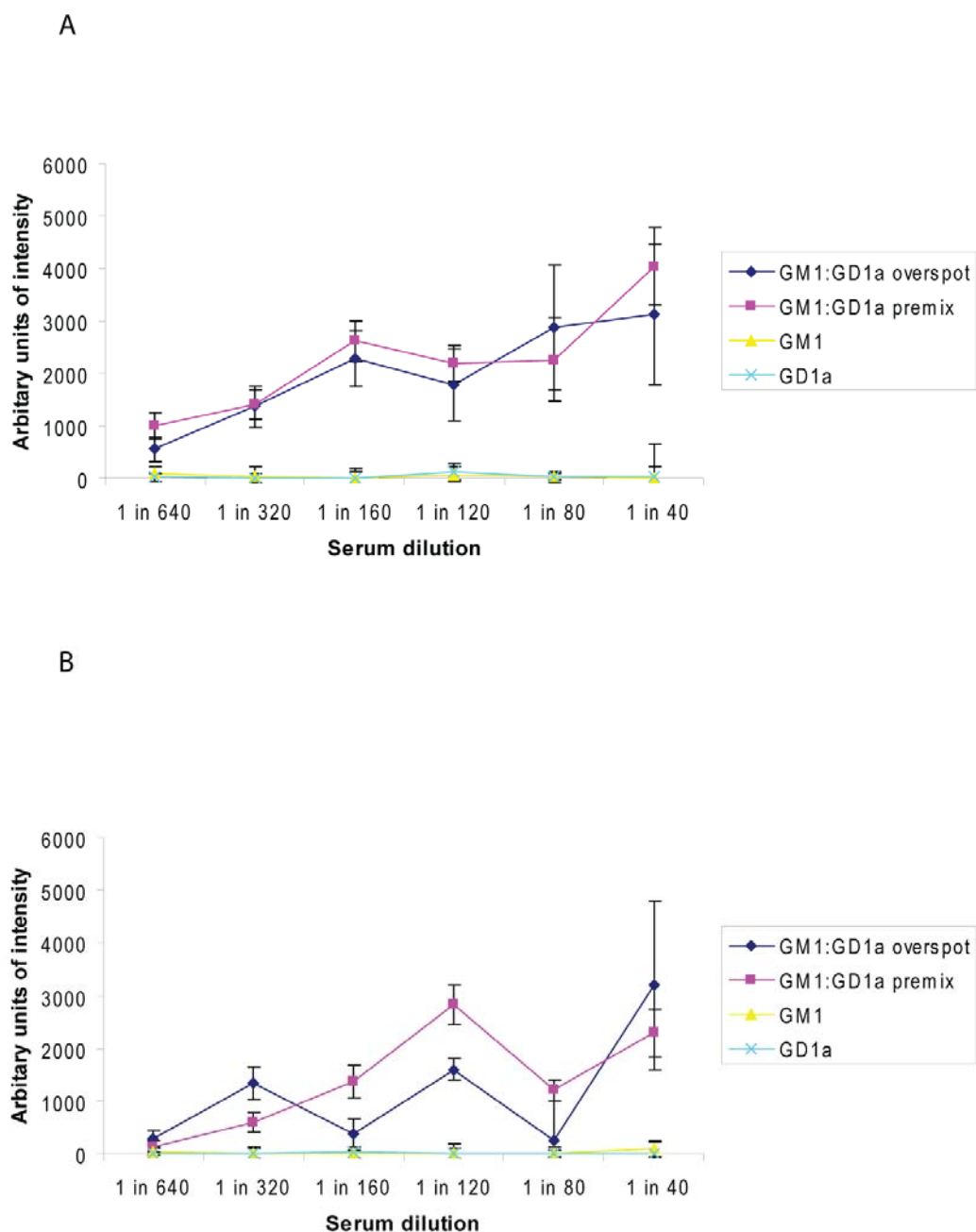


Figure 6.12. Intensity of binding of serum P.J to lipids with and without dye (amido black).

Complexes were printed using two mechanisms. They were either mixed together before application (premix) or simply printed one on top of the other (overspot)

A. Preferential binding of sera to the complex created by GM1 and GD1a.

B. Preferential binding of sera to the complex created by GM1 and GD1a. In (B) all lipids have had amido black dye added.



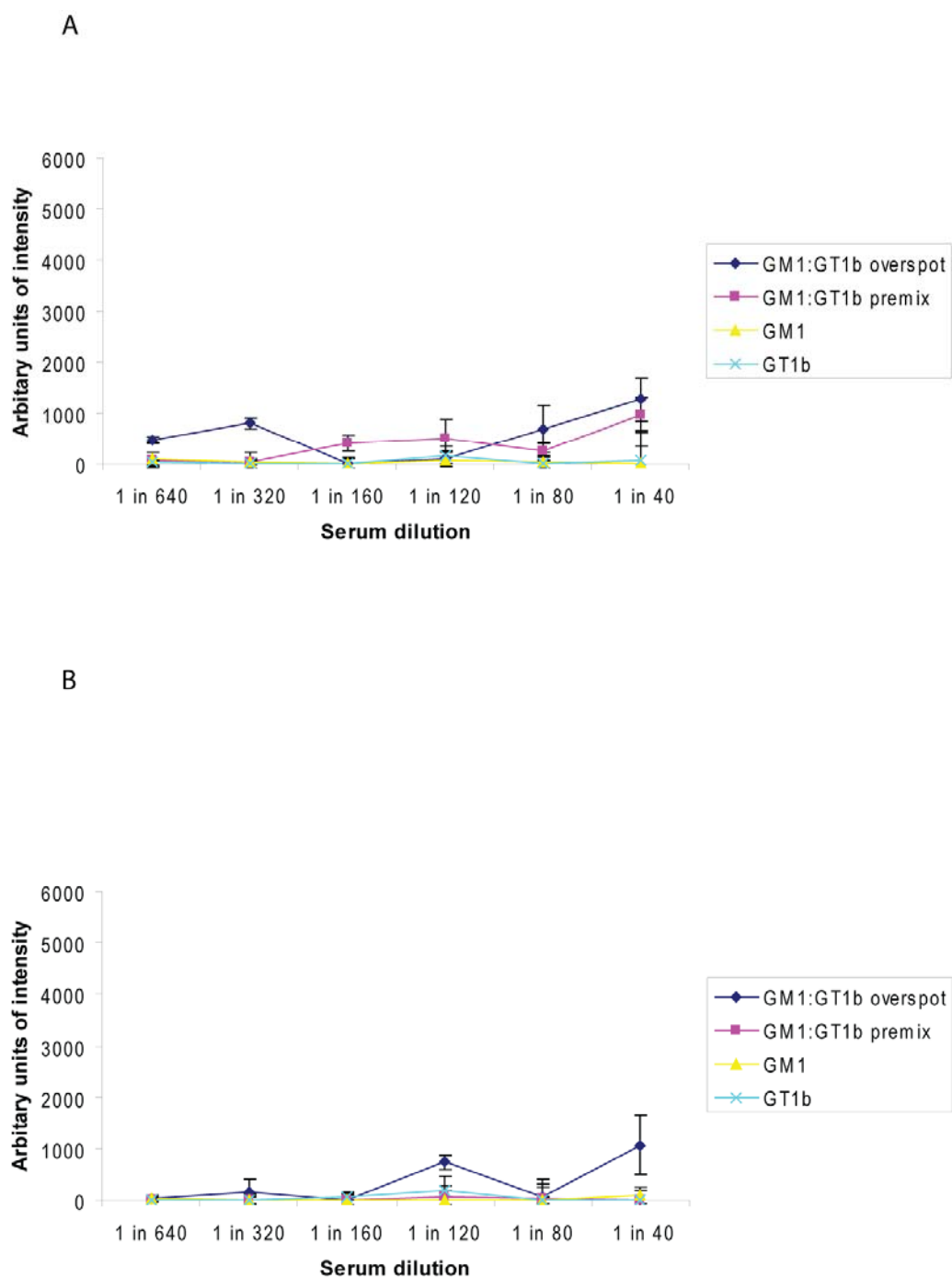


Figure 6.13 Intensity of binding of serum P.J to lipids with and without dye (amido black). Complexes were printed as before.

A. Preferential binding of sera to the complex created by GM1 and GT1b.

B. Preferential binding of sera to the complex created by GM1 and GT1b. In (B) all lipids have had amido black dye added.

### 6.3. Discussion and further work:

Whilst I have improved on this lipid microarray protocol to some degree numerous questions remain unanswered. For example when creating complexes by spotting one lipid over another will inhibitory complexes still be apparent? When I used this technique of printing one lipid over another to create a lipid complex with the combinatorial array technique the effect of inhibitory partnerships on lipid binding was lost (see Chapter 2, Figure 2.10). If this attenuation is lost in the microarray platform then an option would be to use the microarray method to interrogate a large number of potential antigens initially and then revert back to ELISA or the combinatorial array technique to investigate lipid and lipid complex specificities of individual antibodies.

In addition there is considerable variation even when printing the positive control sample, AlexaFluor 647. This sample is used commonly as a positive control in other experiments in which protein is arrayed. This variation in printing of AF 647 may simply be as a result of the age/state of the arrayer. Furthermore the arrayer used for these experiments is not specifically designed to work with solvent based substrates. Therefore using an arrayer specifically designed to work with methanol may prove advantageous. Indeed the RASOR group at the University of Glasgow have purchased a new arrayer with this purpose in mind.

## Chapter 7. Final Discussion

The combinatorial array has allowed us to investigate the binding specificities of a wide variety of anti-lipid antibodies in three different cohorts. We were able to identify that 42/180 GBS sera demonstrated IgG that was reactive to a wide variety of glycolipids. Interestingly over half of these seropositive sera (26/42) demonstrated anti-ganglioside complex antibodies by using a modified version of Kaida's definition of anti-ganglioside complex antibodies, (i.e. where intensity for IgG directed against the complex is more than the sum of the intensities of IgG directed against the two individual contributory lipids)(Kaida et al. 2007). This is in direct contrast to the CIDP cohort where only 4/19 seropositive sera demonstrated anti-ganglioside complex antibodies using this definition.

Why anti-glycolipid complex antibodies should be more frequent in the acute neuropathy, GBS compared to the more chronic CIDP is, as yet, unclear. This may relate to pathogenesis of the neuropathy in question. It is well described that molecular mimicry between *campylobacter jejuni* lipooligosaccharides (LOS) and peripheral nerve glycolipids plays a role in the pathogenesis of GBS(Ang et al. 2004). The *c. jejuni* LOS contain glycolipid-like moieties that can determine the specificity of anti-glycolipid antibodies. Importantly immunisation of *c. jejuni* LOS to animal models induces anti-glycolipid antibodies and a clinical picture similar to GBS demonstrating the pathogenic potential of such antibodies(Yuki et al. 2004). One study has investigated serum anti-ganglioside complex antibodies from GBS patients with a history of antecedent infection with *c. jejuni* (Kuijff et al. 2007). They then demonstrated that these anti-ganglioside complex antibodies cross-reacted with LOS from autologous *c. jejuni* isolates which would suggest that these antibodies were induced by the *c. jejuni* infection. There is no firm evidence for a role of molecular mimicry in the

pathogenesis CIDP as yet and perhaps this may explain the relative absence of anti-glycolipid complex antibodies in this disease.

The combinatorial array yielded interesting results in the Multiple Sclerosis (MS) cohort. Whilst serum anti-lipid IgG specificity was similar between the MS and control groups, the CSF profiles differed demonstrating a CSF specific IgG anti-sulfatide response. This led us to collaborate with two independent groups who had developed complementary techniques of creating recombinant IgG from the CSF of MS patients. Similar binding specificities were observed for Scottish derived CSF, recombinant IgG created using single cell PCR from American MS patients and recombinant IgG created from German MS patients via gel electrophoresis, a series of digestions and mass spectrometry. This has allowed us to identify for the first time at least some of the binding specificity of the oligoclonal bands in MS. *Cis*-interactions may prove to be of vital importance here as the complex created by sulfatide and sphingomyelin appeared to inhibit binding of these anti-sulfatide antibodies which did not bind intact myelin in culture based systems.

However it is worth noting that assays of anti-lipid antibodies are fraught with difficulty with potential variability being introduced at numerous points. As illustrated aptly in Chapter 3 with GT1a, antigen source and purity is of vital importance. Timing of samples taken in relation to illness is also a likely important factor e.g. it is well described in GBS that anti-glycolipid antibodies decline with time. Other issues such as oxidation of stored lipids are likely to be relevant. With the use of the combinatorial array in conjunction with ELISA, it is become increasingly apparent that detection methods used for anti glycolipid antibodies will have a degree of inherent variability. This is something which is well described when comparing ELISA results between laboratories with high inter-laboratory variation reported (Willison et al. 1999).

Future studies which will be carried out as a result of this work include:

1. Combinatorial array screen of GBS & CIDP cohorts using additional glycolipid antigens:

The combinatorial arrays employed to investigate the anti-glycolipid repertoire in both GBS and CIDP are limited and have some notable omissions namely SGPG, sulfated glucuronyl paragloboside, which is potentially a target antigen in neuropathy. The sulfoglucuronyl moiety appears to be an essential part of the epitope for anti-SGPG antibodies(Ilyas et al. 1991). This moiety is present on several other nervous system glycoconjugates including MAG, neural cell adhesion molecule and P<sub>0</sub>, the major glycoprotein of peripheral nervous system myelin(Kruse et al. 1984)(Bollensen et al. 1987). Therefore antibodies to SGPG may have multiple potential target antigens and could interfere with the structural integrity of myelin through a variety of mechanisms. Ilyas et al reported that 13/53 (25%) of GBS patients demonstrated anti-SGPG antibodies with 8 demonstrating IgM antibody and 5 demonstrating IgG antibody. Despite the subsequent negative study by Yuki et al this antigen may be of relevance in GBS (Yuki, Tagawa & Handa 1996a) as discussed by Ilyas et al. The authors mention that when serum from paraproteinemic neuropathy patients with anti MAG/SGPG antibodies is injected into feline peripheral nerve extensive myelin destruction is observed which is not dissimilar to that seen in acute GBS. (Hays et al. 1987)(Willison et al. 1988). Furthermore inoculation of rabbits with SGPG with appropriate adjuvants produced a clinical picture of neuropathy, led to the development of anti-SGPG antibodies with neurophysiological studies revealing reduced conduction velocities and conduction block.

A further more extensive combinatorial array investigating the anti-glycolipid responses of the GBS cohort is currently underway. A limitation of

my study was the low number of healthy controls. This has been addressed in this current study with a higher number of healthy controls available.

A major failing of my GBS study is the lack of adequate neurophysiological data. In total neurophysiological data was unavailable for 31/180 patients. Furthermore neurophysiological studies were either equivocal or unavailable for 29/42 patients with positive anti-glycolipid antibodies. AMAN and AMSAN are distinct clinical phenotypes yet both are associated with antibody directed against the same antigen, GM1. This ganglioside is present in equal amounts in motor and sensory nerves. 3/4 patients with AMAN demonstrated anti-glycolipid reactivity specifically to GM1 in a variety of patterns including complex dependent, attenuated and complex independent patterns. However there were no patients with AMSAN in the cohort to compare with the AMAN binding profiles. It would be interesting to interrogate a well characterised cohort of AMAN and AMSAN patients to establish if there is indeed a difference in glycolipid complex specificity which may help explain why sensory nerves are spared in AMAN.

### 3. Elucidation of the role of lipid reactive recombinant antibodies

It is important to determine the biological relevance of both the sulfatide reactive and unreactive recombinant antibodies derived from MS patients. A pathological role for anti-sulfatide antibodies in MS has been suggested. In the nervous system sulfatide is mostly present in oligodendrocytes and Schwann cells. It is first detected during oligodendrocytes differentiation and is upregulated before the oligodendrocytes wrap myelin around axons which may suggest that in addition to its role in maintaining the structural integrity of myelin it has other roles (Eckhardt 2008). Other roles remain to be elucidated fully. Inconsistencies have been described with the administration of sulfatide to animal models of MS with both suppression and augmentation of disease reported (Jahng et al. 2004)(Kanter et al. 2006).

For the first time we have demonstrated that a proportion of recombinant IgG from CSF of MS patients demonstrate lipid binding properties with sulfatide/sulfatide complexes being the primary lipid antigen. The next step is to investigate the role of these antibodies. To do this a variety of techniques can be used. Rat and mouse myelinating culture systems provide a suitable platform to start these investigations. Our preliminary studies reveal that these lipid reactive recombinant IgG behave differently to monoclonal anti-sulfatide antibody 04. The 04 antibody has a characteristic lipid specificity on the combinatorial array binding to the complex of sulfatide and sphingomyelin and binds to intact oligodendrocytes in a rat myelinating culture system. Interestingly the recombinant IgG derived from MS patients did not reproduce these binding profiles. The introduction of a demyelinating injury to these culture systems may reveal the target lipid antigen (s). Furthermore the co-administration of lipid reactive recombinant IgG with appropriate adjuvants to animal models of MS may help elucidate the role further. Clearly this should be performed in conjunction with parallel studies involving the 04 antibody.

### 3. Further development of the microarray technique to allow wider scale screening of lipid and protein antigens

Of course as aforementioned we have only screened small arrays consisting of 9- 10 lipids and their one to one combinations. We have not taken into account the inherent variability of lipid antigens for example it is known that there is variability in the degree of hydroxylation, length of fatty acid chains and degree of esterification within myelin based lipids(Norton 1977). This adds another level of complexity to our continued search for target antigens.

In addition it is known that lipids reside in membrane microdomains with proteins as well as other lipids. The possibility that antigenic targets consisting of several lipids and proteins exists, and deserves further exploration. Interrogation of such target antigens is not feasible on the

current platform and demands a further development of a microarray technique which allows the investigation of both protein and lipid antigens. We have made a small step towards the development of such a technique.



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